Hansenula polymorpha

as a new promising organism for high temperature alcoholic fermentation of lignocellulose sugars

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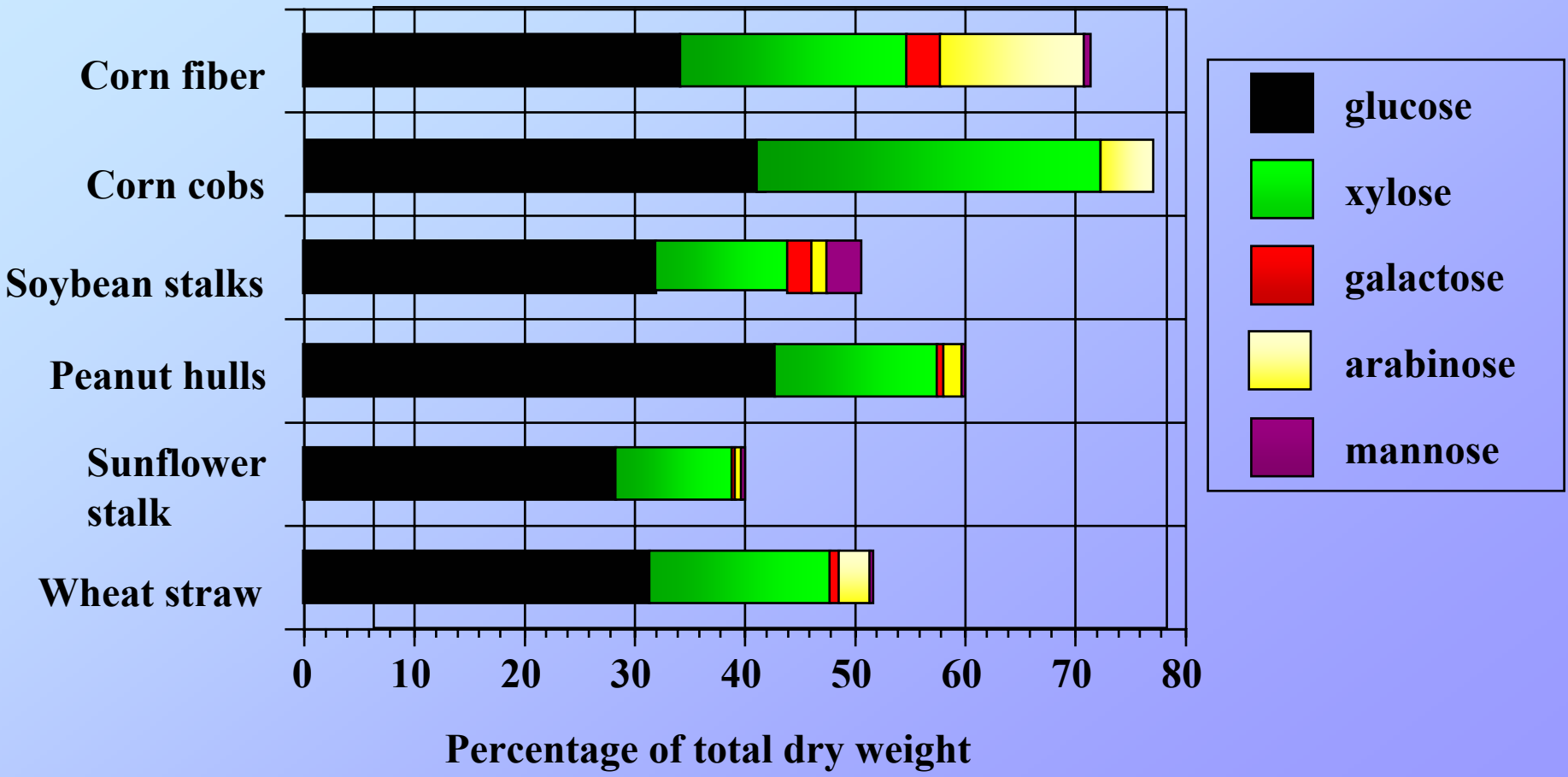
Glucose and xylose are the main components of agricultural residues

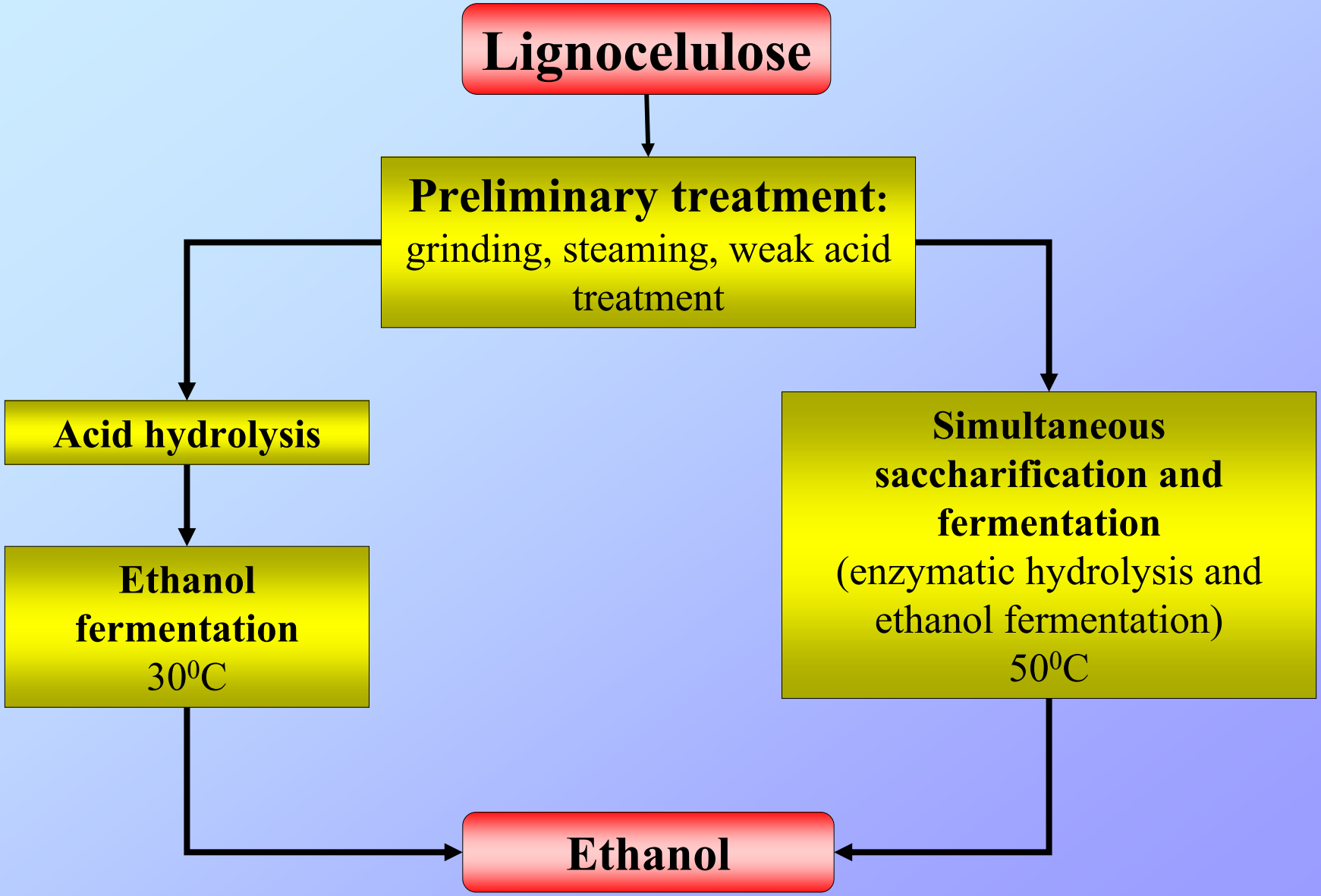


Agricultural and wood wastes can be major income sources

- Harvest residues are significant resources.
- Processing residues are particularly economical.
- Fermentation to ethanol is technically feasible.
- The conversion process is becoming economical.
- Bioconversion adds value to farm products.

Reasons for research

- Xylose is a prevalent sugar in fast growing hardwoods and agricultural residues
- Its utilization is essential for the conversion of lignocellulosic residues to fuels and chemicals
- Yeasts are among the best organisms for producing ethanol and other products by fermentation

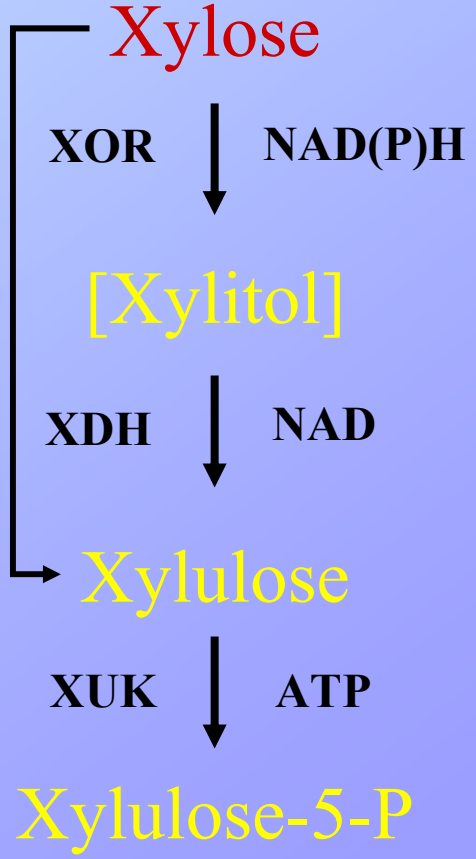

Lignocelulose
Preliminary treatment:
grinding, steaming, weak acid treatment
Acid hydrolysis
Ethanol fermentation
30⁰C
Simultaneous saccharification and fermentation
(enzymatic hydrolysis and ethanol fermentation)
50⁰C
Ethanol

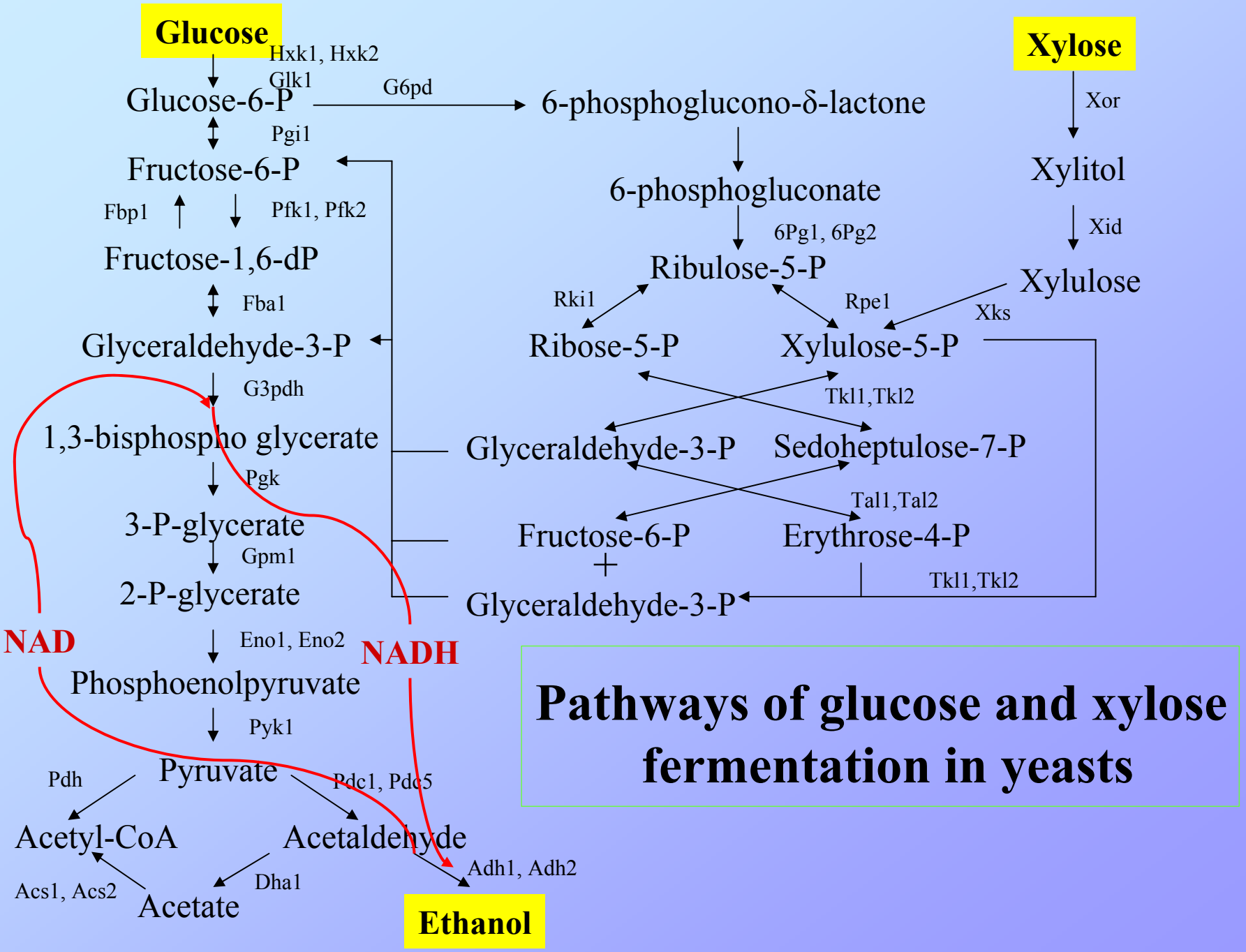
Reasons for study of the yeast *Hansenula polymorpha* as a fermenter of lignocellulose sugars

- ***H. polymorpha*** **is a thermotolerant yeast. Its maximal growth temperature is 49 – 50^{o} C. It is able to ferment xylose to ethanol at elevated temperatures. Efficient fermentation at high temperatures is important for development of the Simultaneous Saccharification and Fermentation (SSF) technology.**

H. polymorpha is among the best yeasts for producing ethanol from xylose

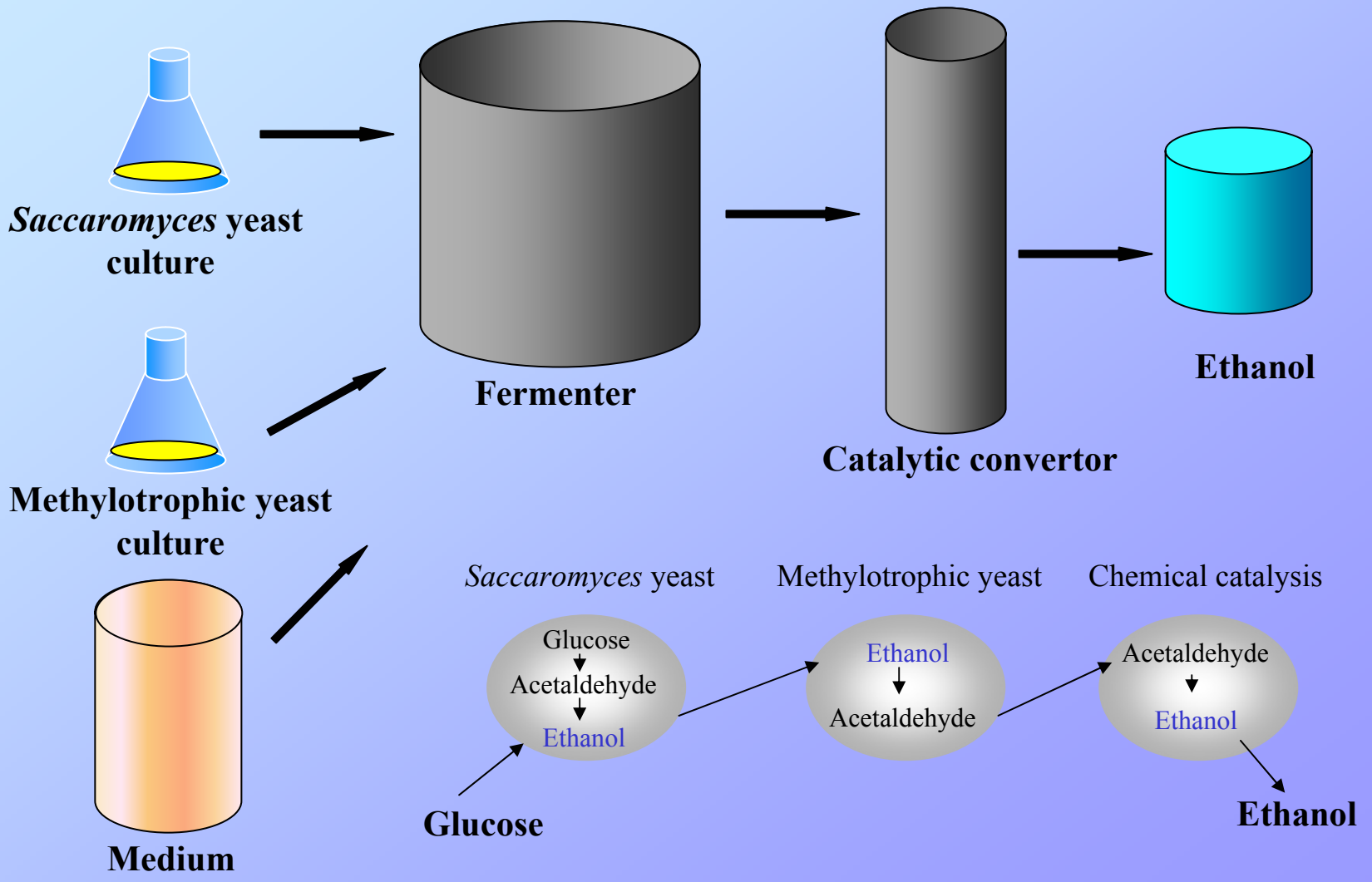
- Xylose is the second most abundant sugar in nature
- This yeasts have a complete xylose metabolic pathway
- They can convert xylose to ethanol



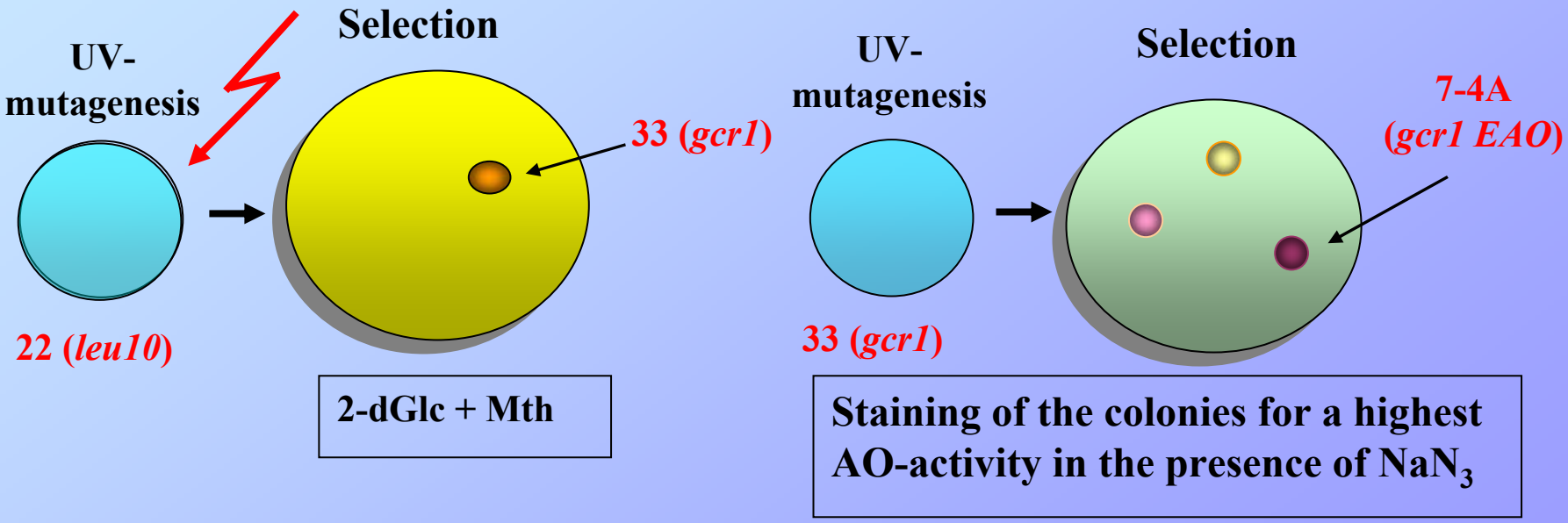


Pathways of glucose and xylose fermentation in yeasts

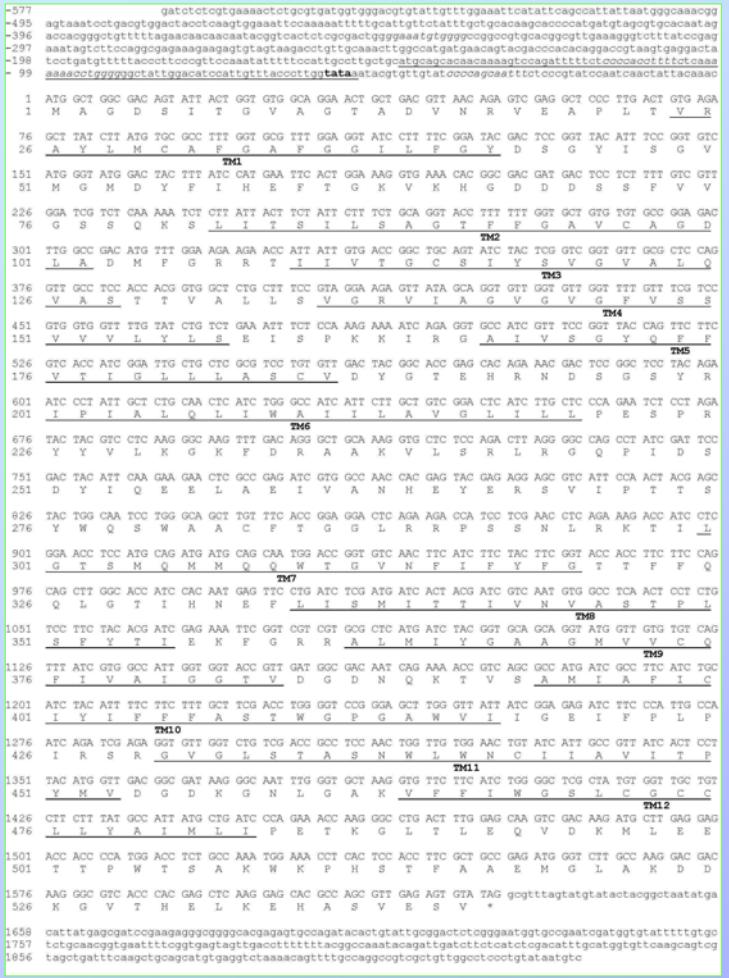
Scheme of mixed culture alcoholic fermetation using *pet⁻ S. cerevisiae* and *gcr1 H. polymorpha*

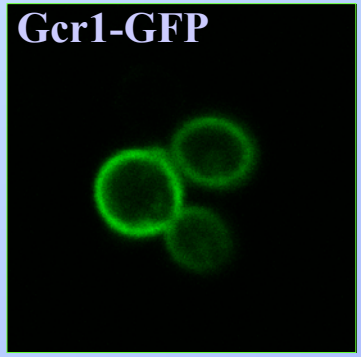


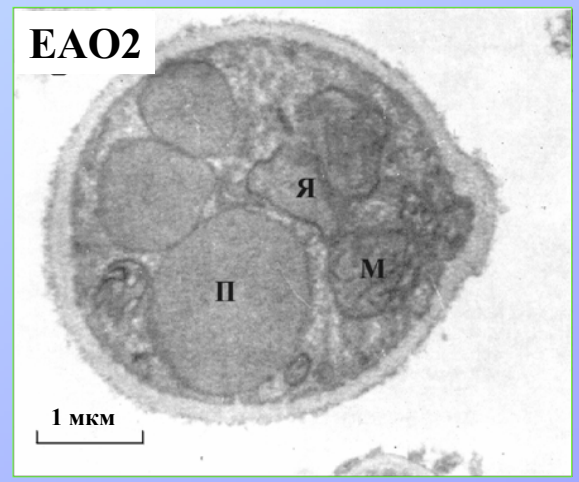
Selection of the *H. polymorpha gcr1* mutants

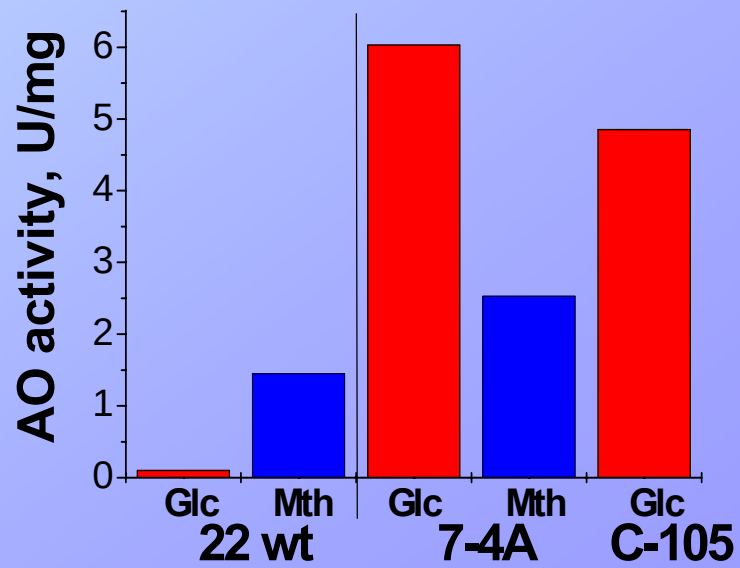


Mutants *H. polymorpha gcr1* defective in glucose repression of alcohol oxidase

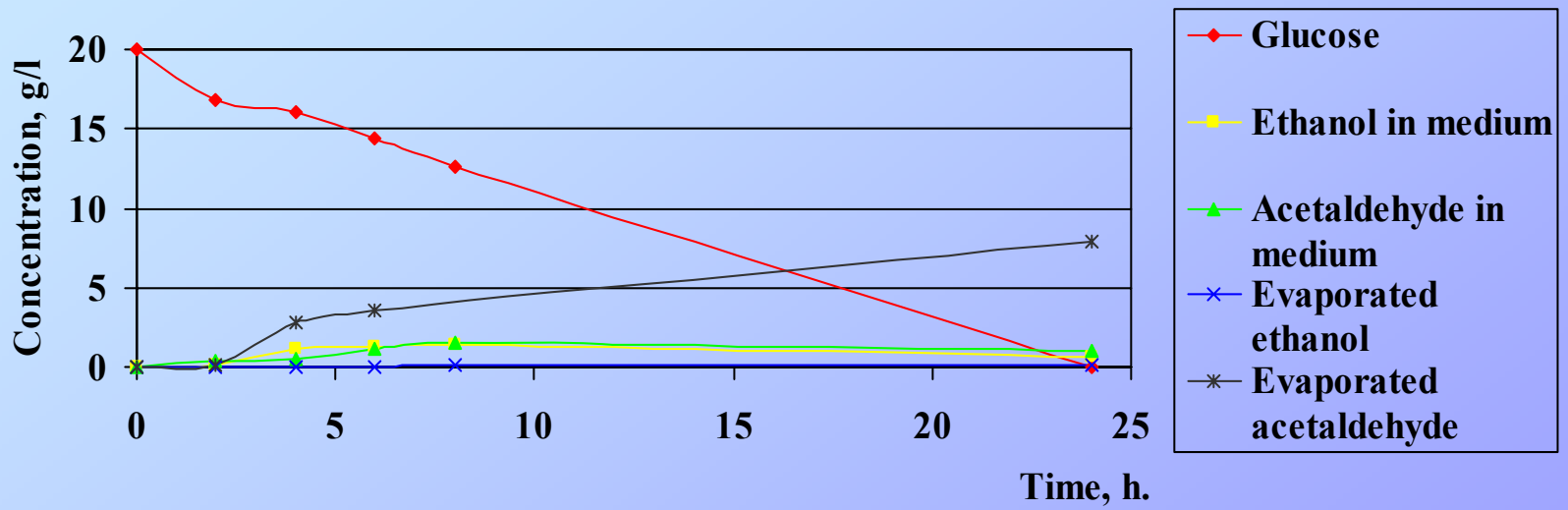


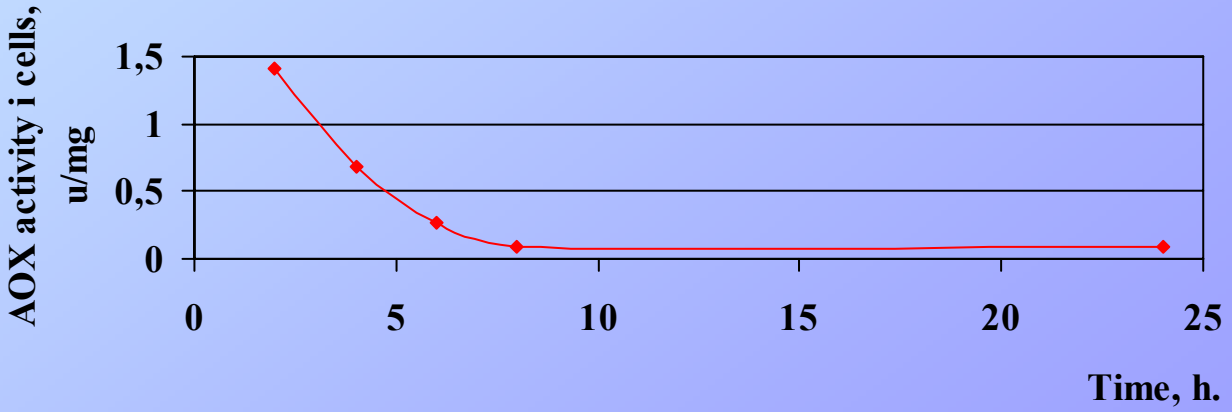




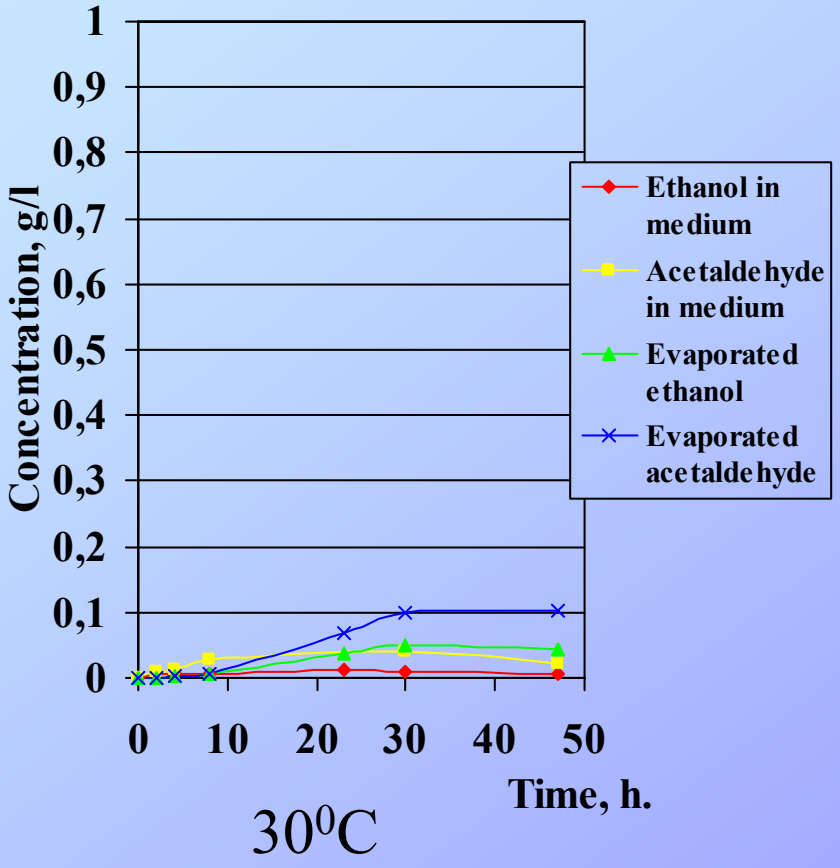


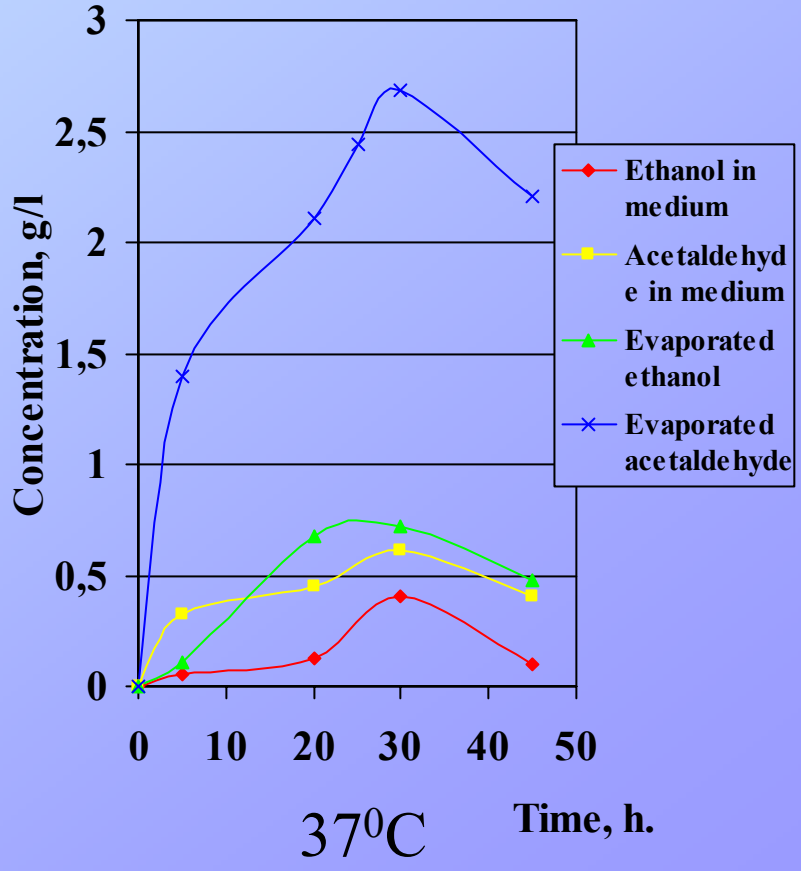
Ethanol and acetaldehyde formation during mixed cultivation of *pet⁻ S.cerevisiae* and *gcr1 H. polymorpha* in glucose (2%) medium



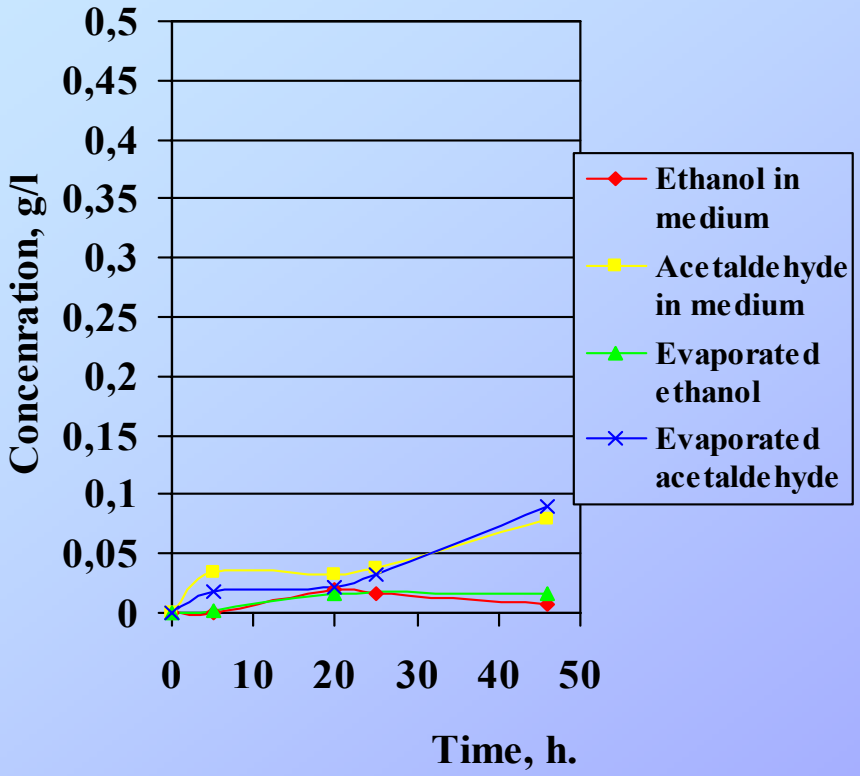


Acetaldehyde formation by *Hansenula polymorpha gcr1* mutant 7-4A in the glucose (2%) containing medium

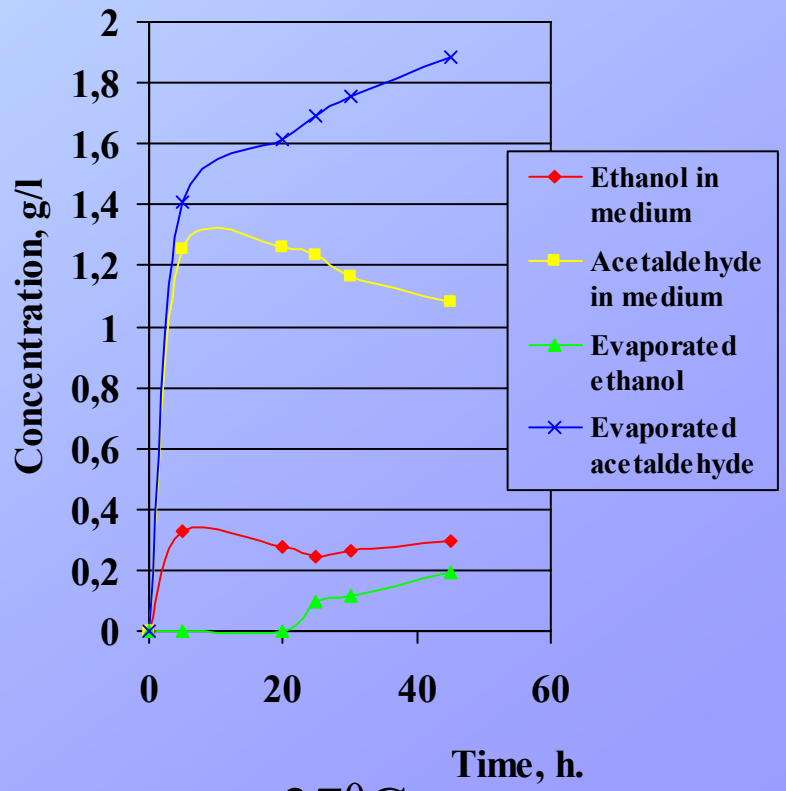




Acetaldehyde formation by *Hansenula polymorpha gcr1* mutant 7-4A in the xylose (2%) medium

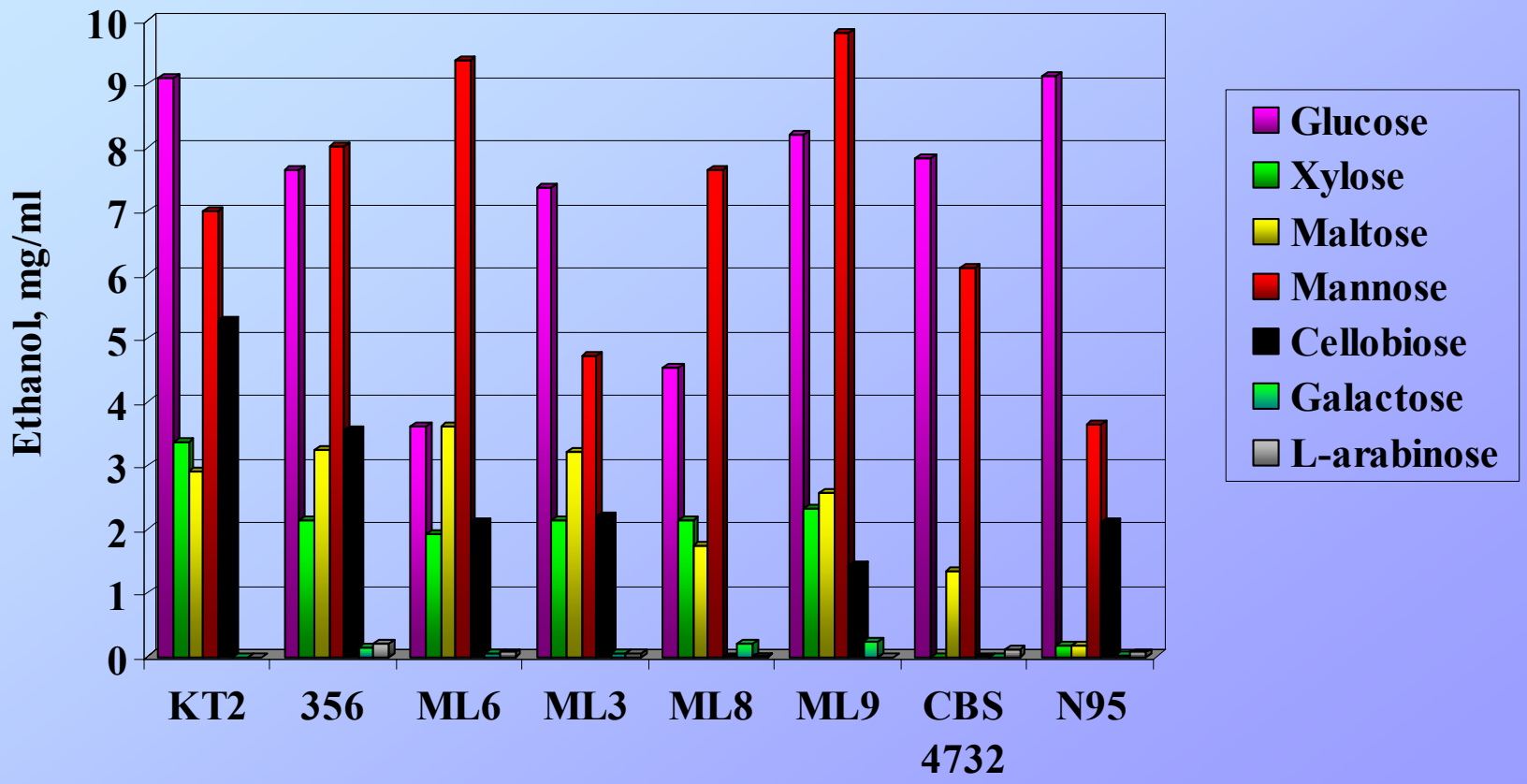


30^{0}C

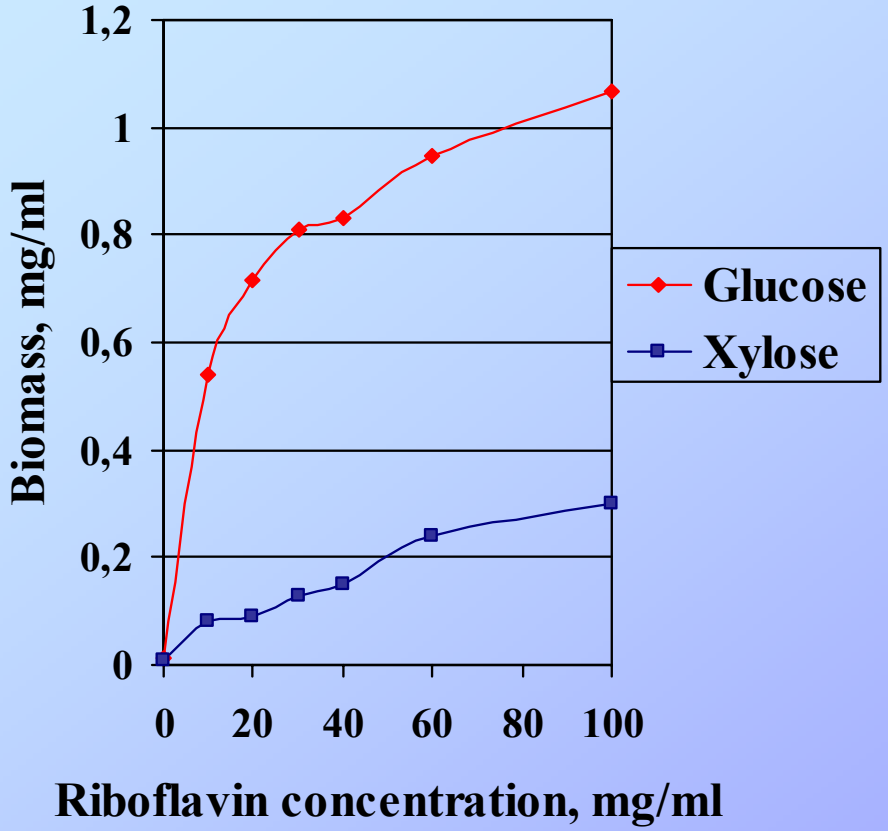


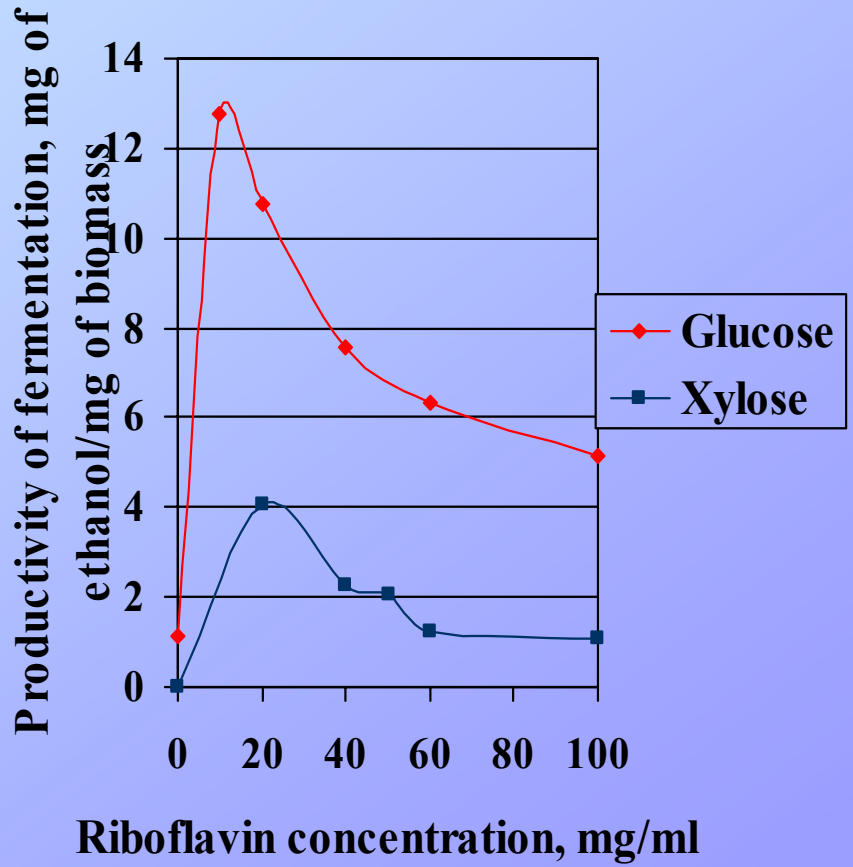
37^{0}C

Ethanol formation by different *Hansenula polymorpha* strains (100h)

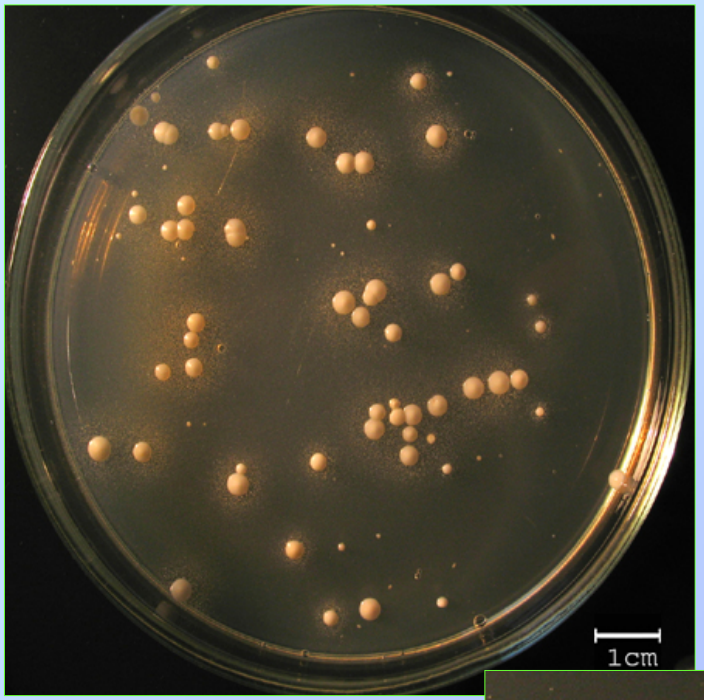


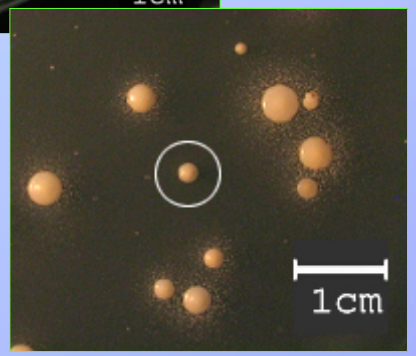
Growth and fermentation of glucose and xylose of riboflavin-deficient *H. polymorpha* strain

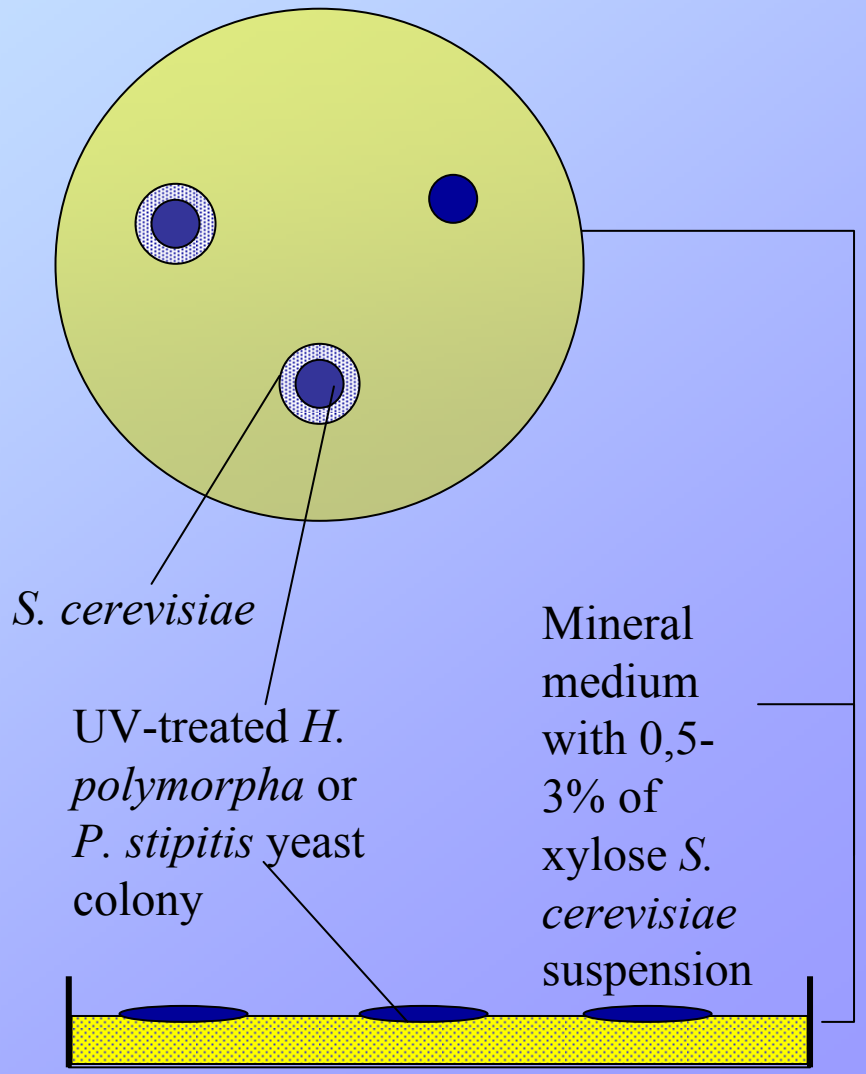




Method of screening of mutants with enhanced fermentation activity

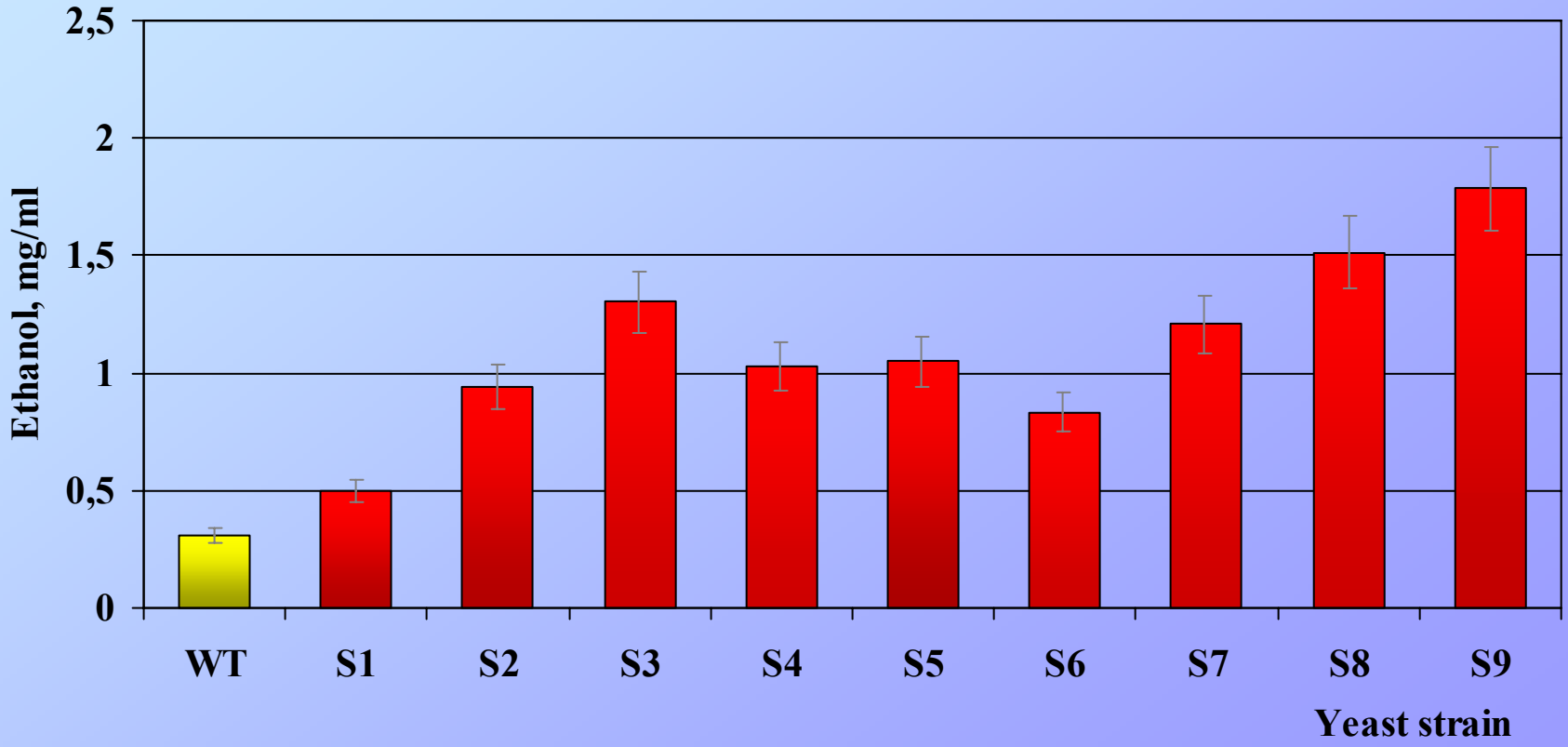






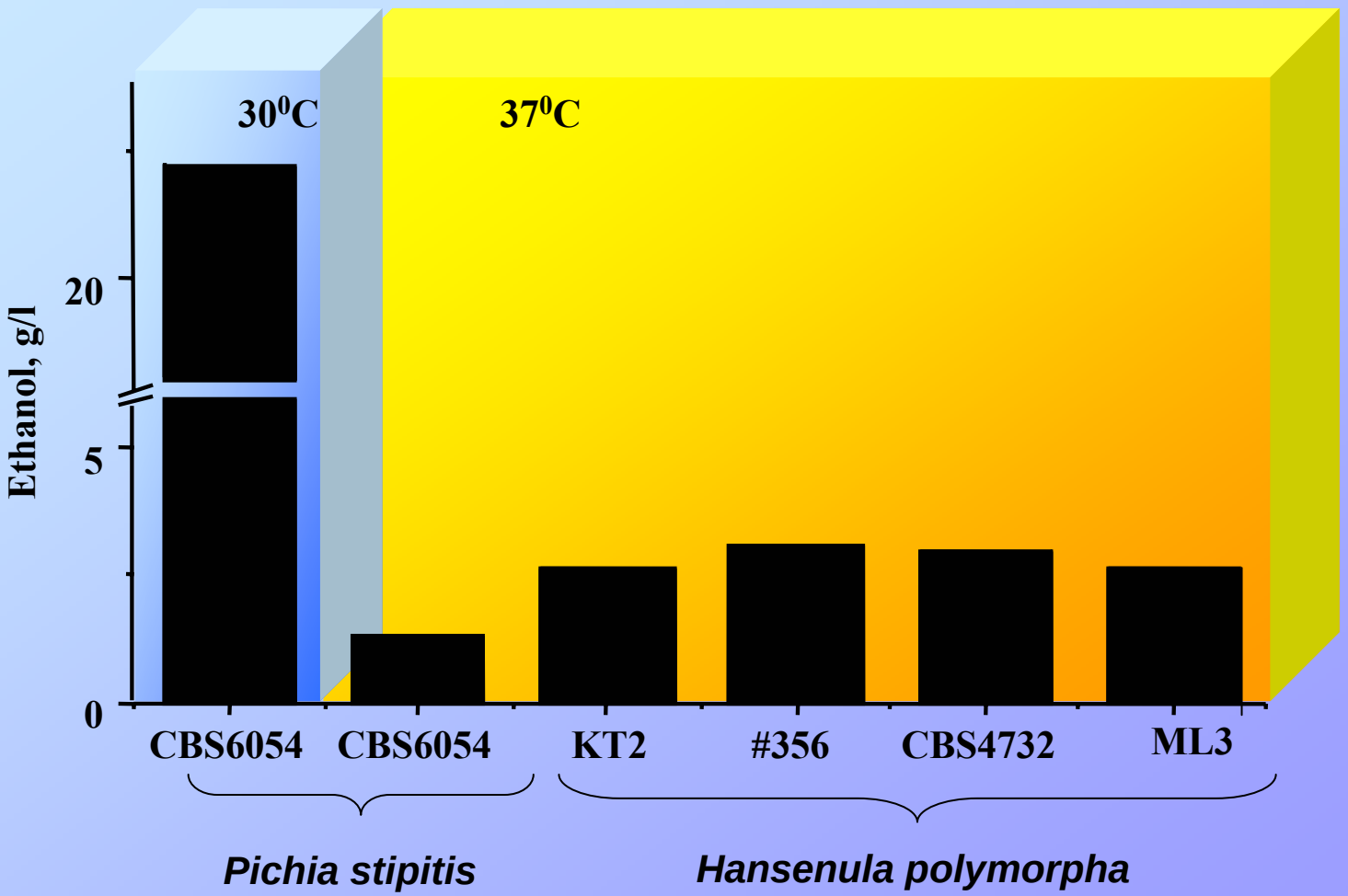
Maximal ethanol production in the medium with xylose (2%) of the *H. polymorpha* wild-type strain and the mutants producing elevated amounts of ethanol

within 60 h. of incubation

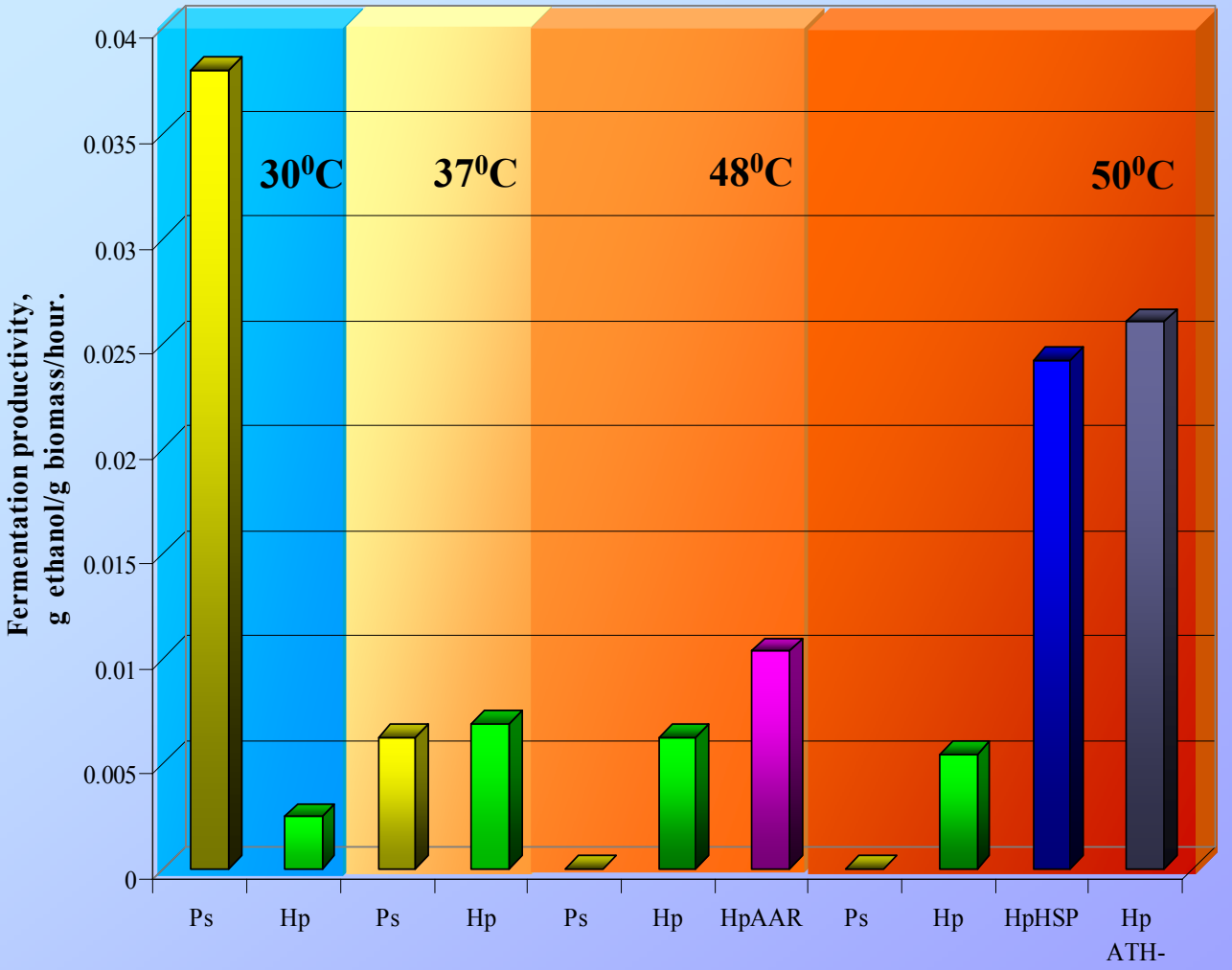


* WT – wild type CBS4732

Xylose fermentation rates by wild type strains of *H. polymorpha* and *P. stipitis*



Xylose fermentation by the *Hansenula polymorpha* and *Pichia stipitis* strains at high temperatures



Ps – *P. stipitis CBS6054*

Hp – *H. polymorpha NCYC495 leu1-1*

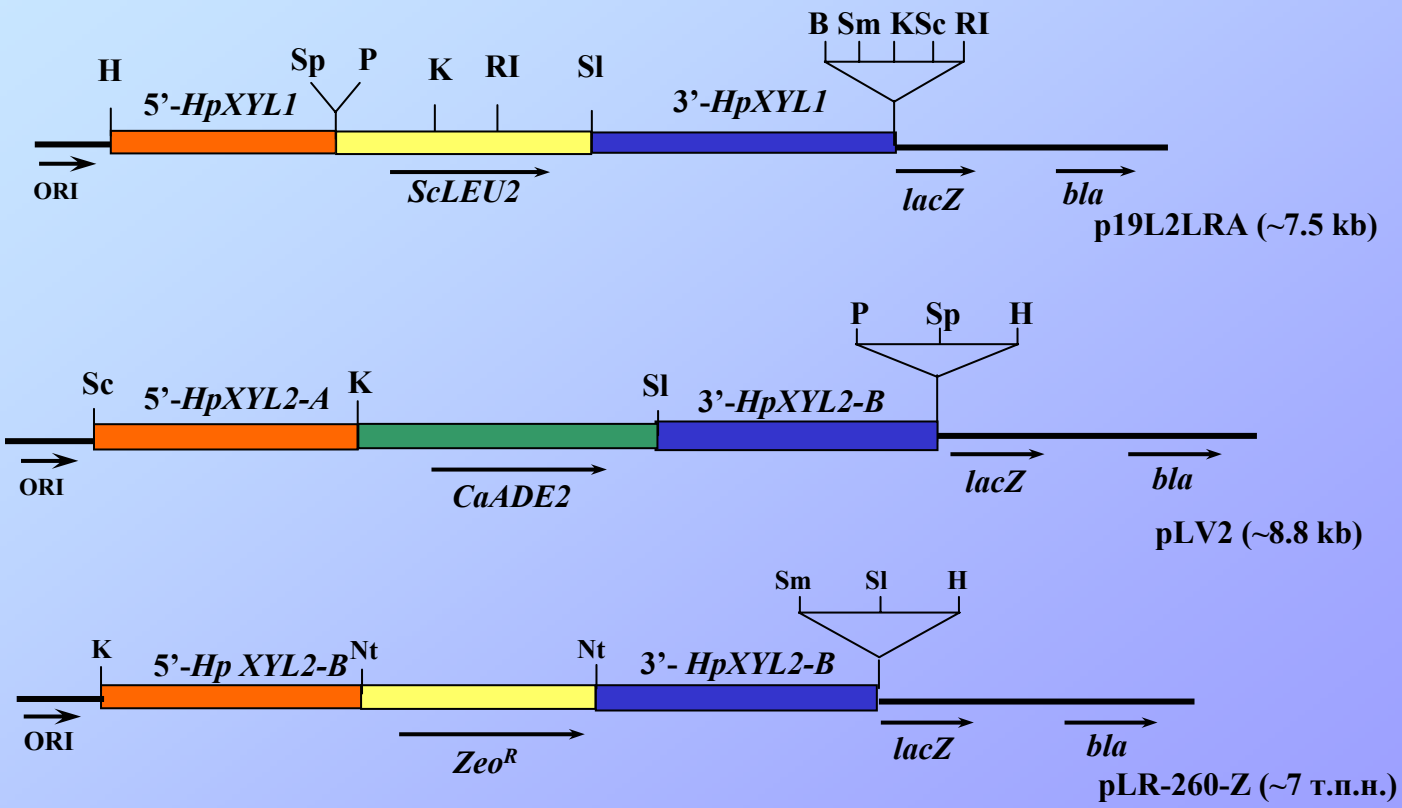
HpAAR - *NCYC495 leu1-1* AllAlcRes

HpHSP - *NCYC495 leu1-1*(*HSP104*)

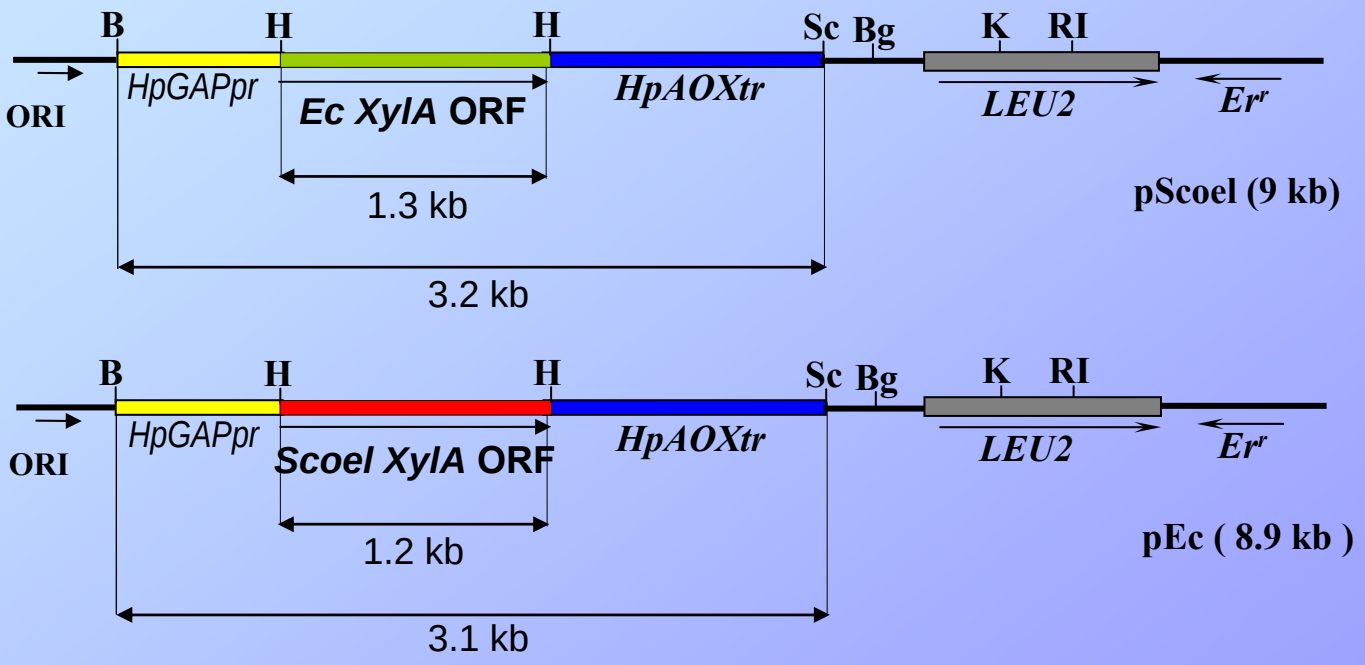
HpATH - - *NCYC495 leu1-1*(*ΔATH1*)

Efficient fermentation at high temperatures is important for development of the Simultaneous Saccharification and Fermentation (SSF) technology.

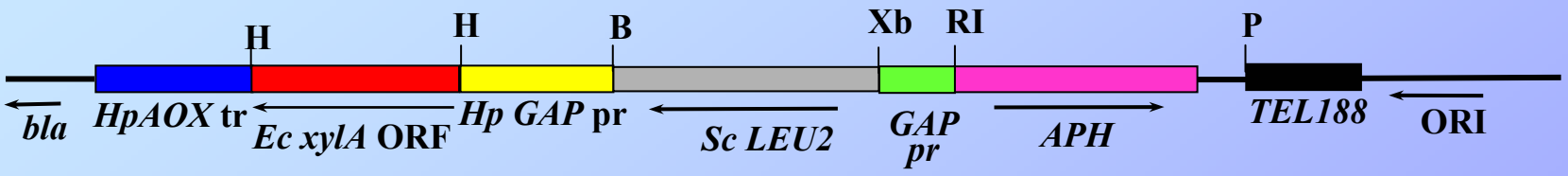
Linear schemes of constructs for deletions of the *XYL1*, *XYL2-A* and *XYL2-B* genes of *H. polymorpha*



Linear schemes of plasmids carrying bacterial *xylA* genes under the *H. polymorpha GAP* promoter



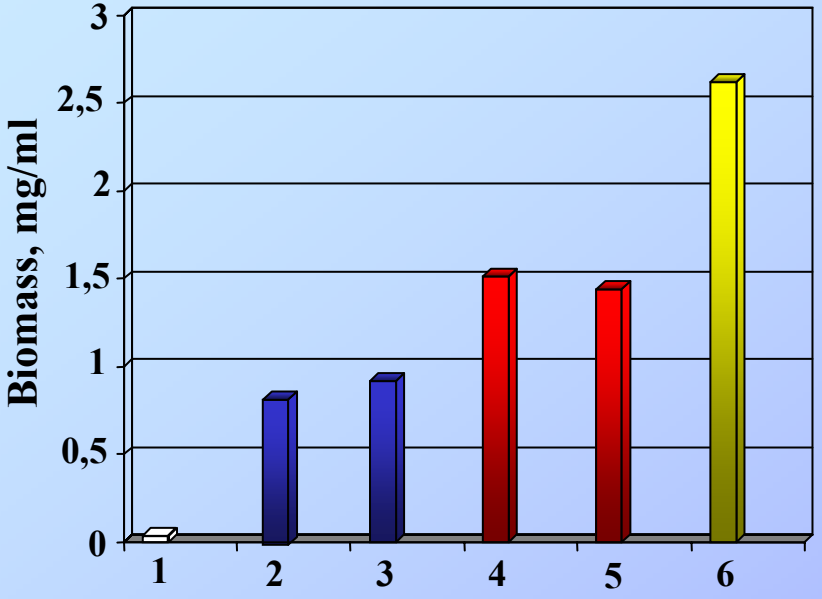
Linear scheme of the plasmid pGLG61_xylAEc for multicopy integration into *H. polymorpha* genome

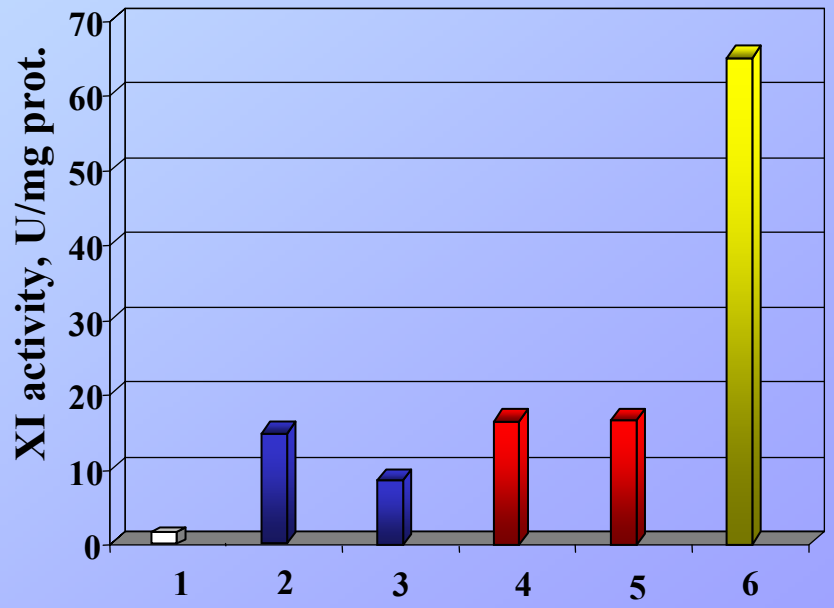


pGLG61_XylAEc ~ 9 т.п.н.

S. cerevisiae **genome fragment containing the** ***LEU2*** **gene is shown as grey box; fragment containing the** ***HpGAP*** **promoter: yellow box; fragment containing the** ***HpAOX*** **terminator: blue box; fragment containing the ORF of** ***E. coli xylA*** **gene: red box; fragment containing the** ***TEL188*** **telomeric fragment: black box; the** ***APH*** **gene of** ***E. coli*** **under control of the truncated** ***GAP*** **promoter of** ***H. polymorpha*** **are shown as violet and green boxes, respectively; the bacterial part: thin line.**
Restriction sites: H, Hind III;; RI, EcoR I; B, BamH I; Xb, XbaI; P, PstI.

Growth and xylose isomerase activity of the *H. polymorpha Δxyl1* transformants containing bacterial *E. coli* and *S. coelicolor xylA* genes





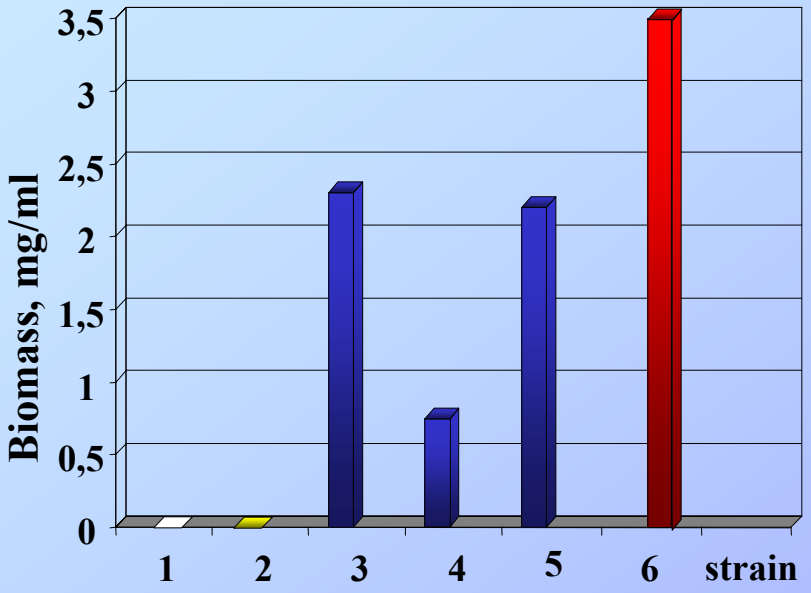
Growth in the liquid minimal medium with 4% xylose as a sole carbon source:

1-*Δxyl1*
***2, 3 - Δxyl1*(pEc)**
***4,5 - Δxyl1*(pScoel)**
6* – CBS4732 *leu2-2

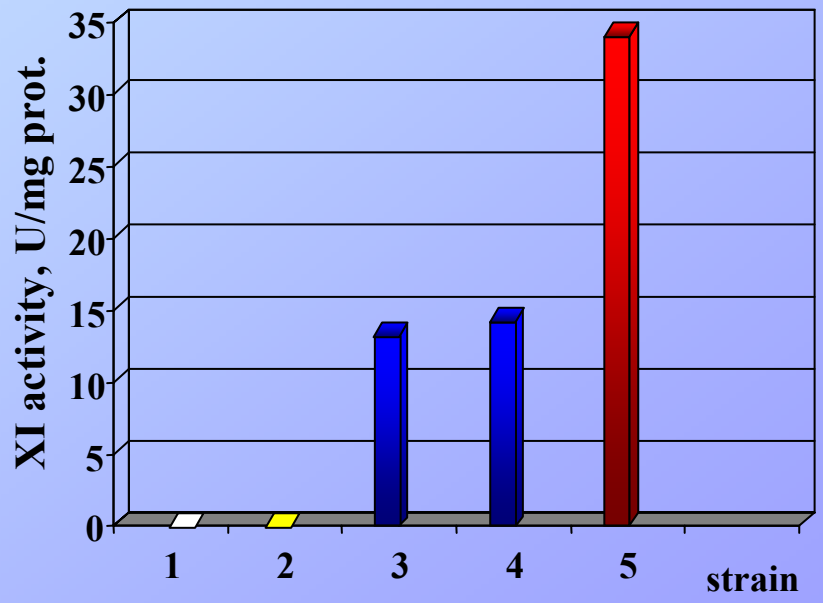
Xylose isomerase activity:

1-*Δxyl1*
***2, 3 - Δxyl1*(pEc)**
***4,5 - Δxyl1*(pScoel)**
6 – E. coli

Growth and xylose isomerase activity of the *H. polymorpha Δxyl1 Δxyl2-A* transformants containing bacterial *E. coli xylA* gene



Growth in the liquid minimal medium with 4% xylose as a sole carbon source:
1 - *Δxyl1*,
2 - *Δxyl1 Δxyl2A*
3, 4, 5 - transformants *Δxyl1Δxyl2A*(pGLG_xylAEc)
6 - CBS 4732 *leu2-2*



The xylose isomerase activity:
1 - *Δxyl1*,
2 - *Δxyl1 Δxyl2A*
3, 4, 5 - transformants *Δxyl1Δxyl2A*(pGLG_xylAEc)
6 - *E. coli*

Isolation of the spontaneous large colonies among the *H. polymorpha* transformants *Δxyl1Δxyl2-A*(pGLG_xylAEc)

YNB +
1 % xylose

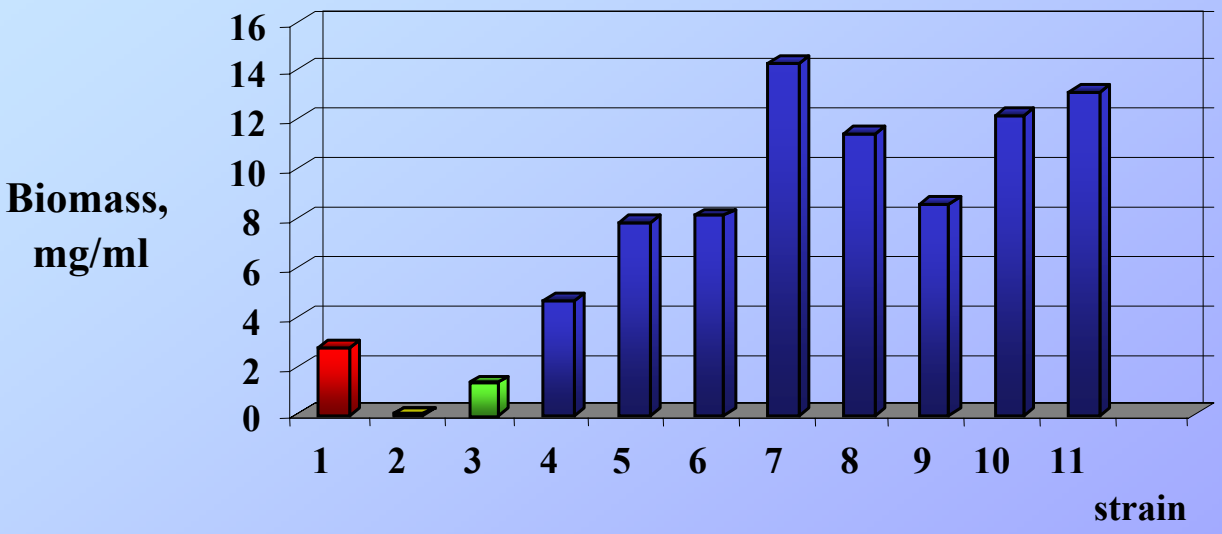
Spontaneous large colonies of the transformants *Δxyl1Δxyl2-A*(pGLG_xylAEc)

Growth analysis of the large colonies in the liquid medium with 2 % xylose

Xylose isomerase activity assay

Determination of ethanol production during xylose fermentation

Growth of the *H. polymorpha* strains in the minimal medium with 2 % xylose at 37° C; 5 days



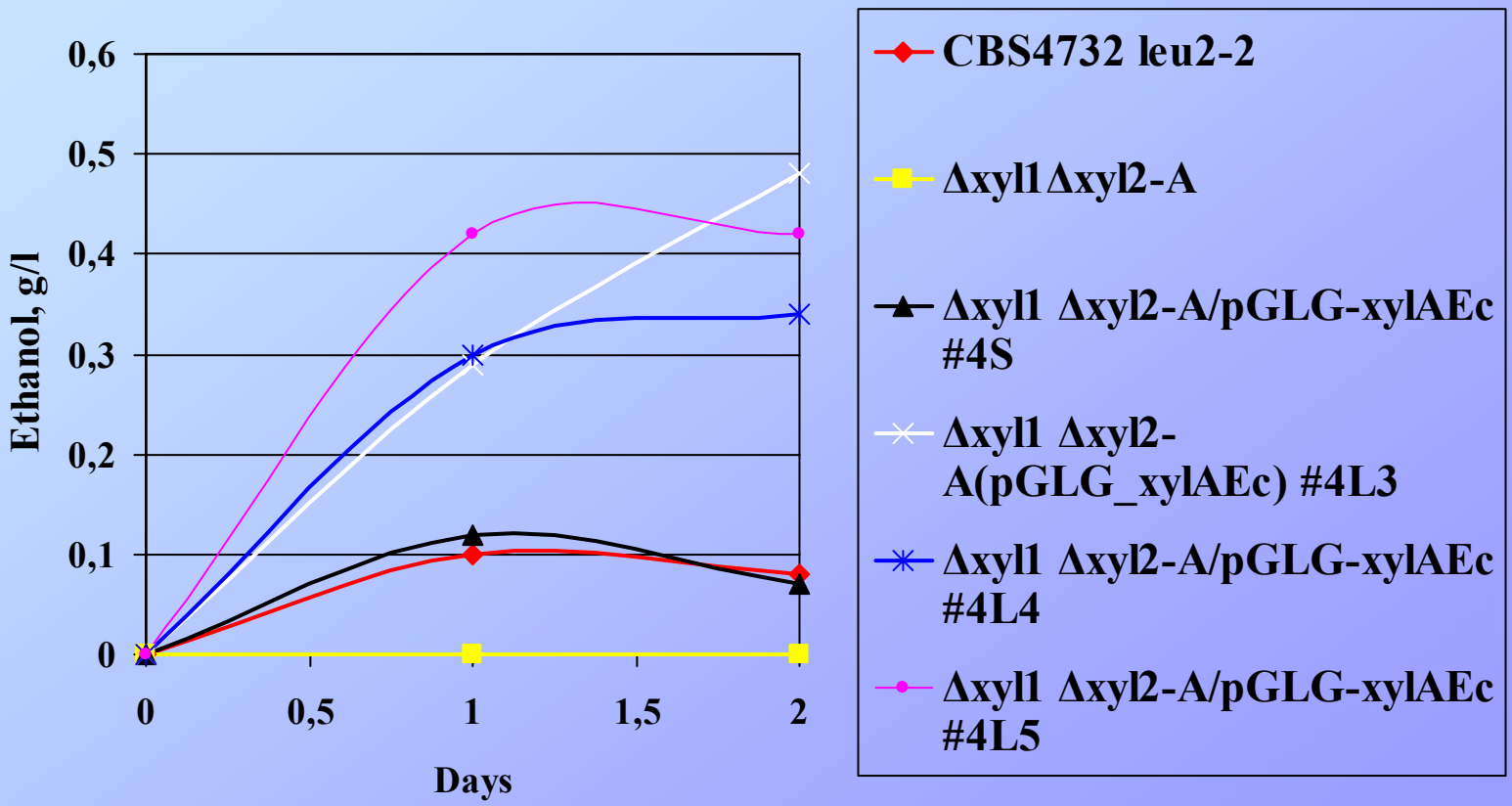
1 - CBS 4732 *leu2-2*

2 - *Δxyl1 Δxyl2-A*

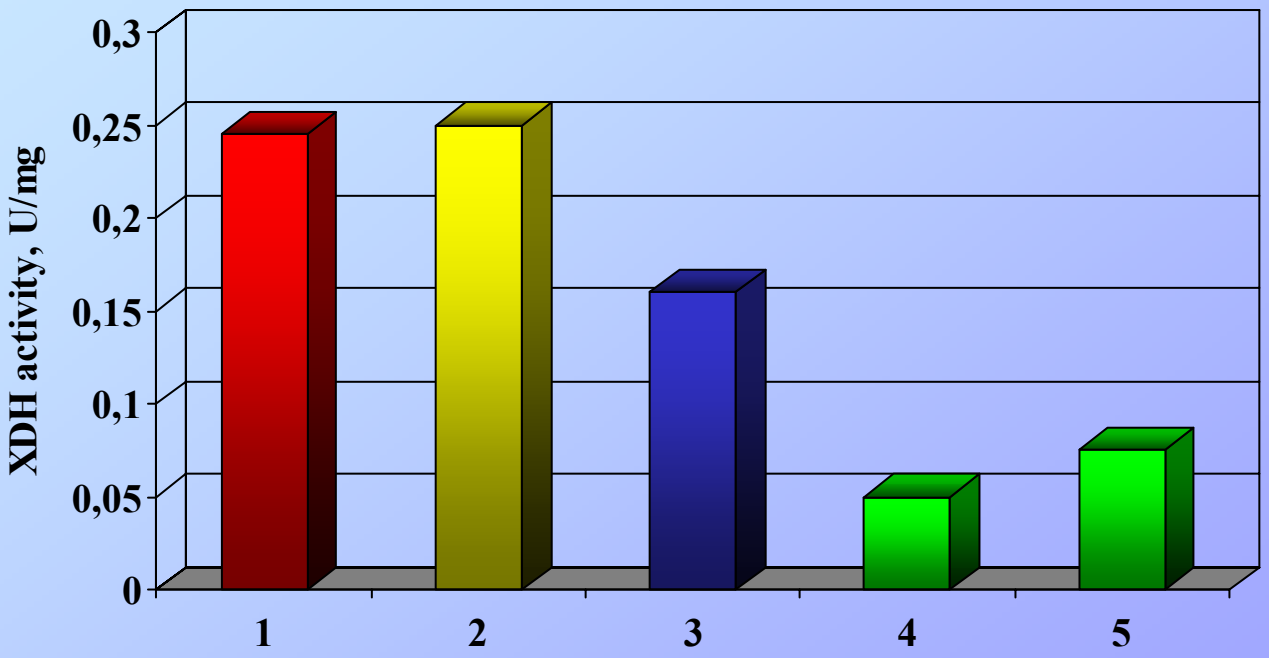
3 - transformant *Δxyl1Δxyl2-A*(pGLG61_xylAEc)

4 – 11 – large clones of the transformant *Δxyl1Δxyl2-A*(pGLG61_xylAEc)

Alcoholic xylose fermentation by the *H. polymorpha Δxyl1 Δxyl2-A*(pGLG_xylAEc) strains at 37^{0}C



Xylitol dehydrogenase activities of the *H. polymorpha Δxyl1 Δxy2-A* and *Δxyl1 Δxy2-A Δxy2-B* mutants

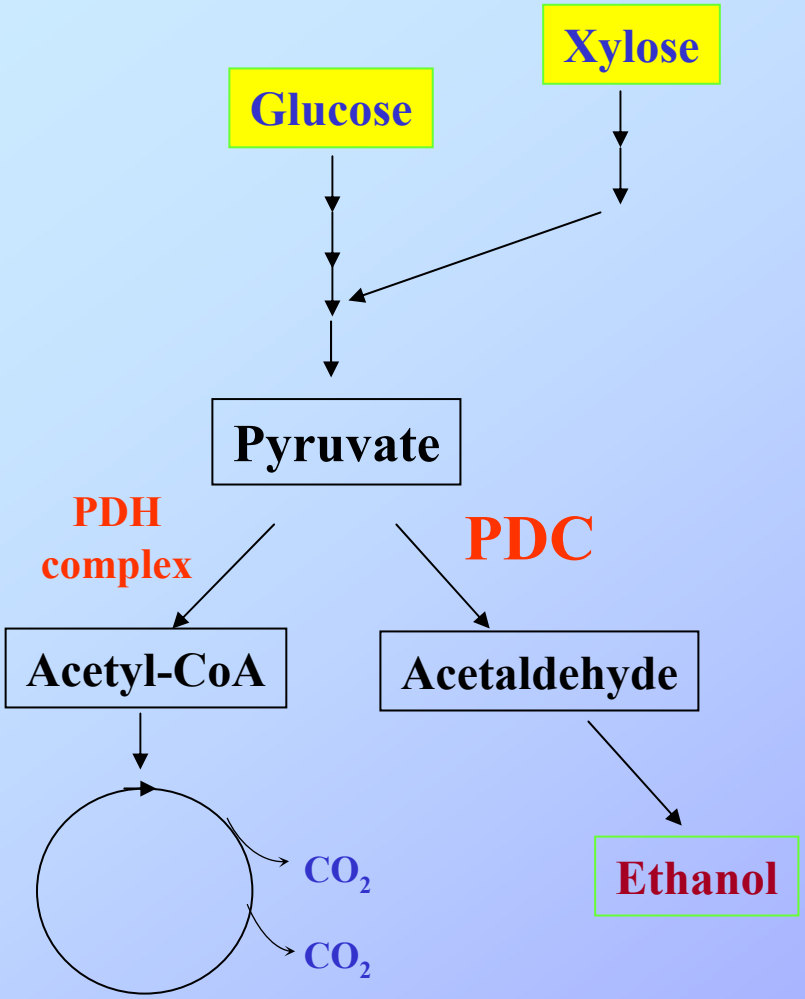


1- *CBS 4732 leu2-2* (control)

2- *Δxyl1*

3- *Δxyl1 Δxy2-A*

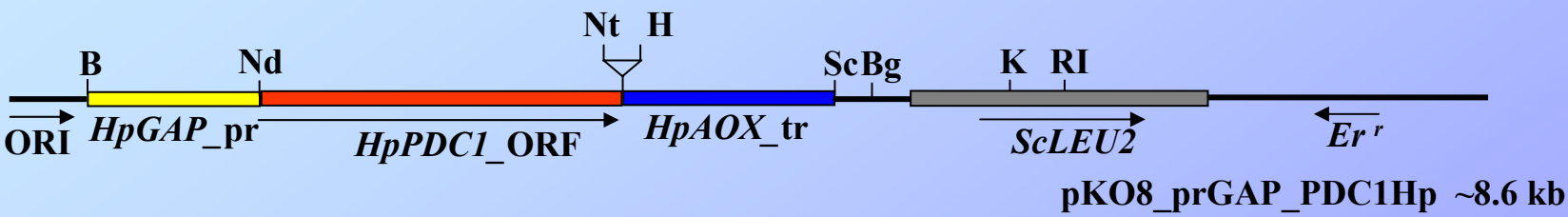
4,5- *Δxyl1 Δxy2-A Δxy2-B*



The aim:

To clone the *H.polymorpha* *PDC1* gene coding for pyruvate decarboxylase and put the gene under the strong constitutive promoter (*HpGAP*pr)

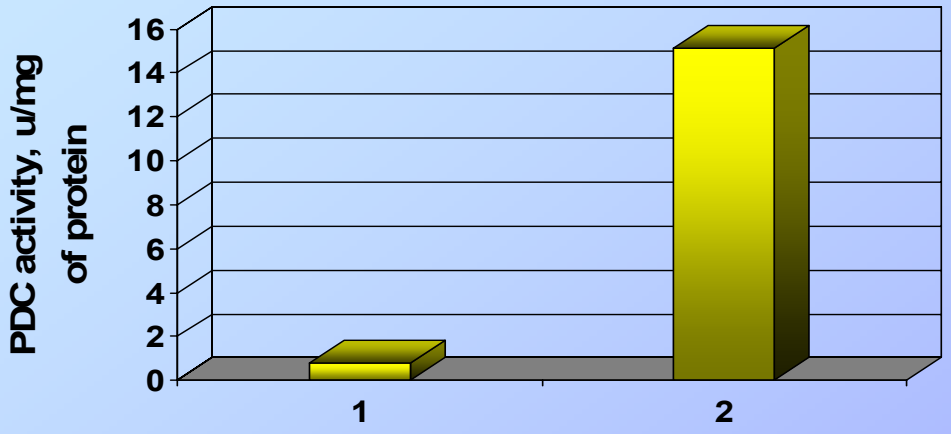
Linear scheme of the plasmid carrying the *HpPDC1* gene under the *HpGAP* promoter

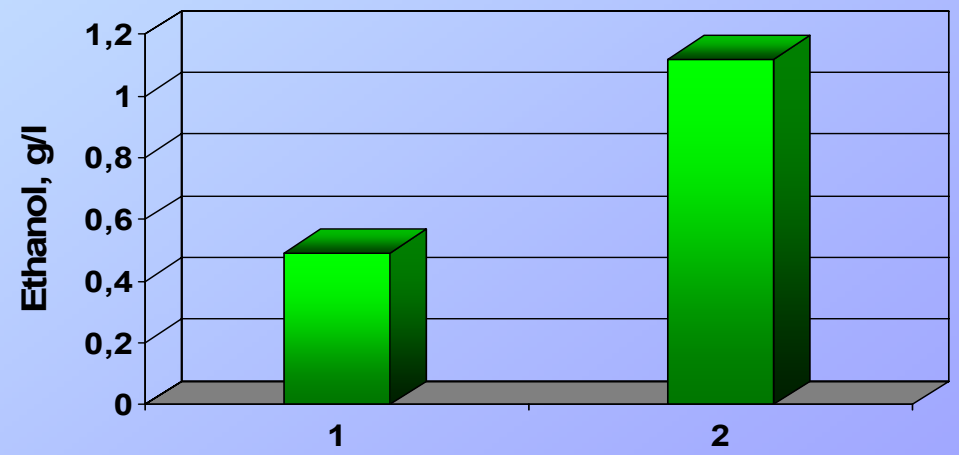


The linear scheme of the plasmid pKO8_prGAP_PDC1Hp (~ 8.6 kb). The *LEU2* gene of *S. cerevisiae* is shown as grey box; fragment containing the promoter of the *HpGAPDH* gene is shown as yellow box; fragment containing the terminator of the *HpAOX* gene: blue box; the ORF of the *H. polymorpha PDC1* gene is shown as red box.
Restriction sites: B, BamH I; K, Kpn I; RI, EcoR I; Bg, BglII; Sc, Sac I; Nd, NdeI; Nt, NotI; H, Hind III.

Effect of the *PDC1* gene overexpression on ethanol production during xylose fermentation

Specific activity of pyruvate decarboxylase (Pdc1p) and ethanol production





1-495-3Leu+

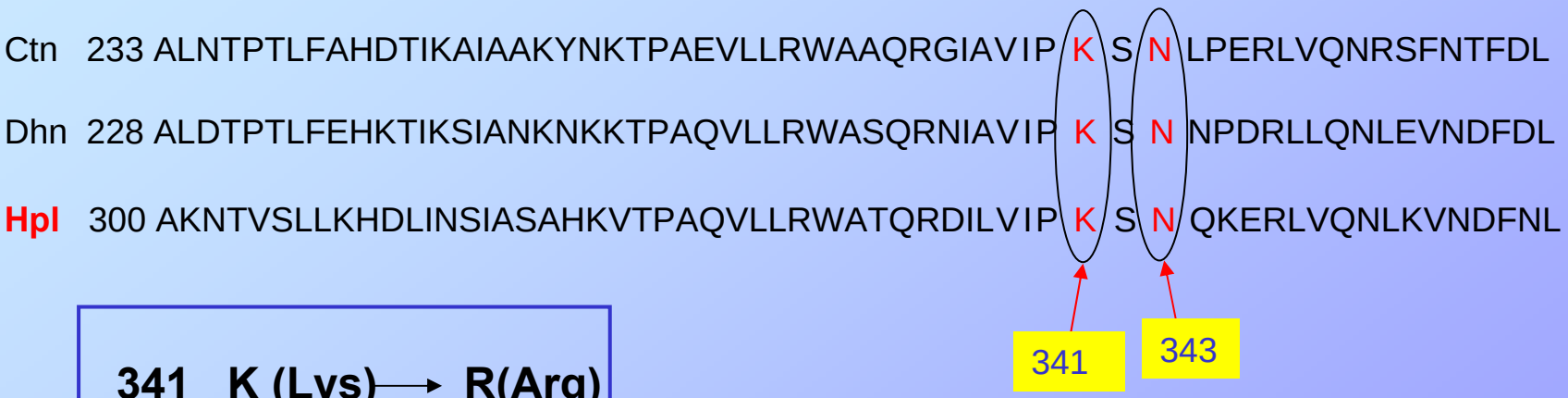
2-495-PDC1Hp-4

YNB + 8 % xylose

37 ºC, 100 rpm

54 hours

The double replacement (Lys → Arg Asn → Asp) results in preference of the modified xylose reductase for NADH over NADPH

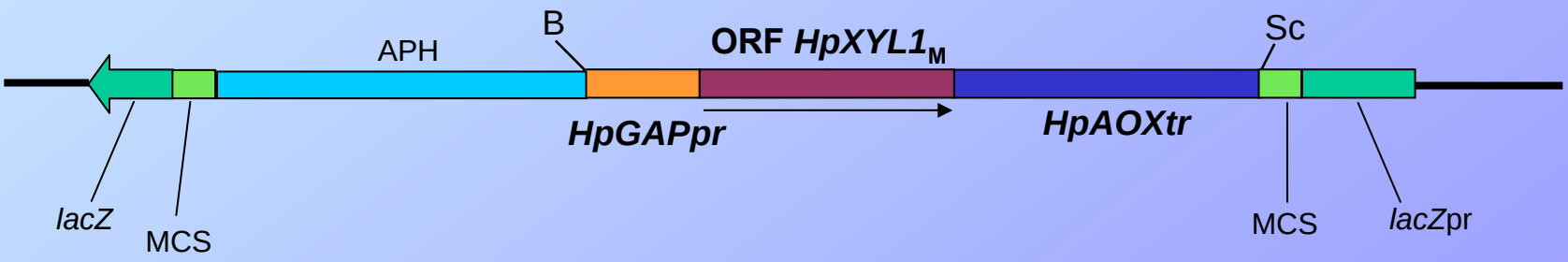


341 K (Lys) → R(Arg)

343 N(Asn) → D(Asp)

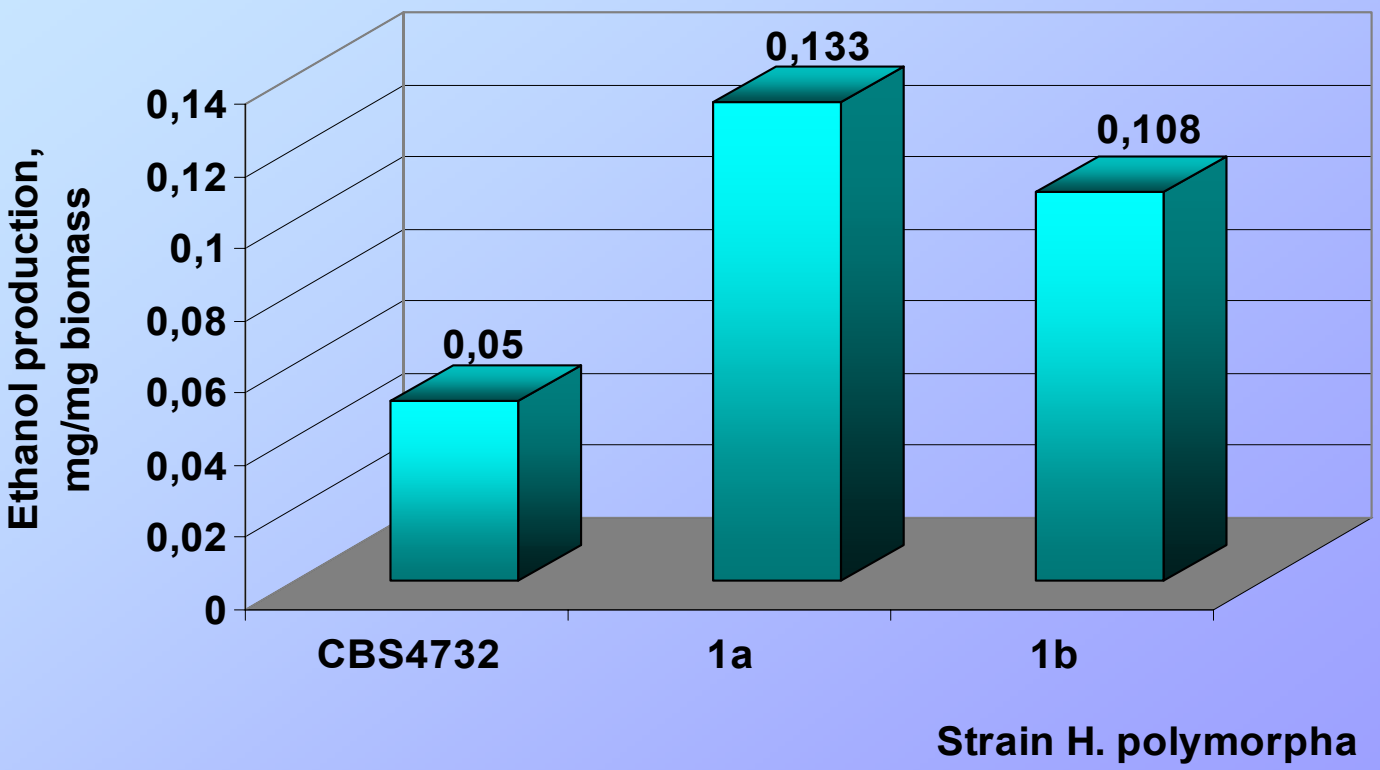
Ctn – *Candida tenuis*
Dhn – *Debaryomyces hansenii*
Hpl – *Hansenula polymorpha*

Linear scheme of the plasmid pX1M carrying the modified *HpXYL1* gene under the *HpGAP* promoter

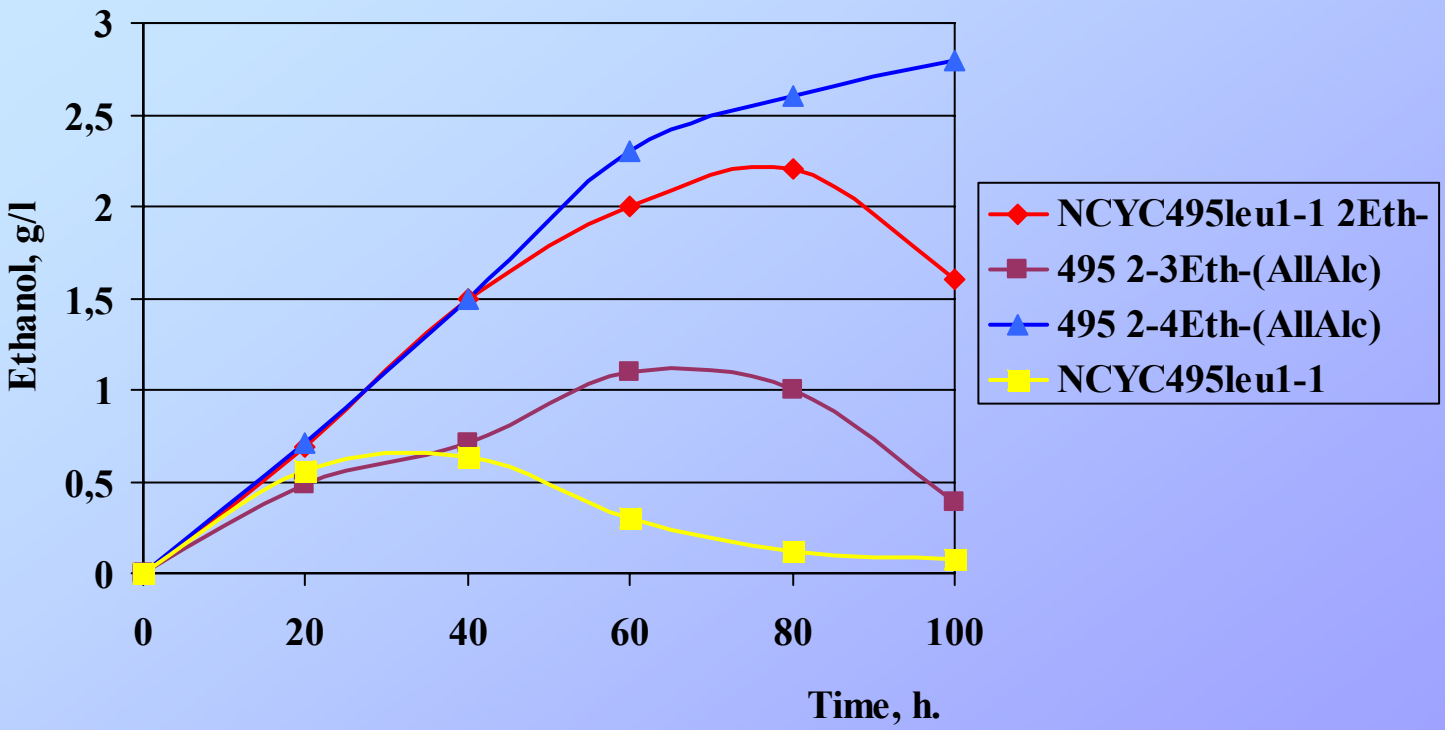


Ethanol production by the CBS 4732 strain and *Δxyl1*(pX1M) transformants;

2 day fermentation, min. medium, 37^0C

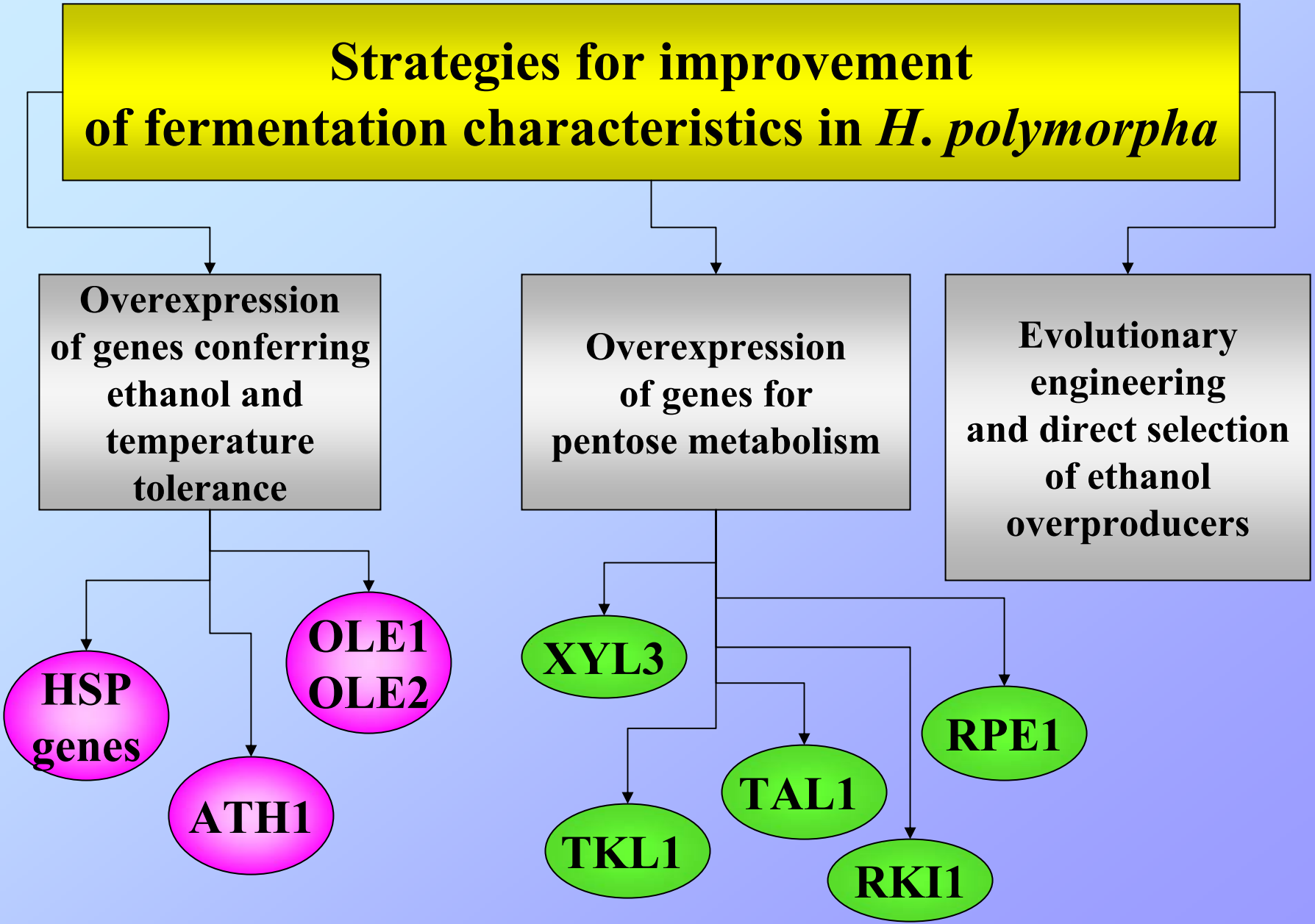


Alcoholic xylose fermentation by the *H. polymorpha* strains unable to utilize ethanol



Minimal medium 12 % xylose; 48ºC.

The strains were selected as resistant to allyl alcohol (0.2мМ).


Strategies for improvement
of fermentation characteristics in H. polymorpha
Overexpression
of genes conferring
ethanol and
temperature
tolerance
Overexpression
of genes for
pentose metabolism
Evolutionary
engineering
and direct selection
of ethanol
overproducers
HSP
genes
ATH1
OLE1
OLE2
XYL3
TKL1
TAL1
RKI1
RPE1

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Thank you

for your attention!